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Review Article

Defining the molecular underpinnings controlling cardiomyocyte proliferation

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Shortly after birth, mammalian cardiomyocytes (CM) exit the cell cycle and cease to proliferate. The inability of adult CM to replicate renders the heart particularly vulnerable to injury. Restoration of CM proliferation would be an attractive clinical target for regenerative therapies that can preserve contractile function and thus prevent the development of heart failure. Our review focuses on recent progress in understanding the tight regulation of signaling pathways and their downstream molecular mechanisms that underly the inability of CM to proliferate *in vivo*. In this review, we describe the temporal expression of cell cycle activators e.g., cyclin/Cdk complexes and their inhibitors including p16, p21, p27 and members of the retinoblastoma gene family during gestation and postnatal life. The differential impact of members of the E2f transcription factor family and microRNAs on the regulation of positive and negative cell cycle factors is discussed. This review also highlights seminal studies that identified the coordination of signaling mechanisms that can potently activate CM cell cycle re-entry including the Wnt/Ctnnb1, Hippo, Pi3K-Akt and Nrg1-Erbb2/4 pathways. We also present an up-to-date account of landmark studies analyzing the effect of various genes such as Argin, Dystrophin, Fstl1, Meis1, Pitx2 and Pkm2 that are responsible for either inhibition or activation of CM cell division. All these reports describe *bona fide* therapeutically targets that could guide future clinical studies toward cardiac repair.

Introduction

Understanding the molecular pathways that underlie exit from the cell cycle may be the key to novel regenerative strategies of the human heart post-injury. The adult mammalian heart is particularly vulnerable to ischemic injury due to irreversible loss of CM that have only a negligible proliferative capacity of surviving CM [1–3]. In contrast, lower vertebrates such as newt and zebrafish, have an amazing ability for cardiac repair through CM proliferation [4]. Intriguingly, myocardial repair based in CM proliferation has been observed in neonatal mice subjected to apical resection [5]. Therefore, the proliferative capacity of CM observed in vertebrate model organisms suggests that the adult mammalian heart could potentially regenerate itself when exposed to appropriate stimuli.

For example, centrosomes are functionally essential components of mitotic spindle poles. Thus, they play an important part in the organization of the mitotic spindle apparatus to faithfully separate the sister chromatids into two daughter cells [6]. Zebrafish and newt CM can maintain the structural and functional integrity of their centrosomes from the neonatal period into adulthood. In contrast, centrosomes in non-regenerative neonatal rat CM functionally disintegrate soon after birth and are sequestered to the nuclear membrane. Centrosomes in adult newt and zebrafish CM, however, function as microtubule organizations centers (MTOC) at the spindle poles during M-phase [6]. Thus, the failure of centrosomes to exhibit proper MTOC activity can contribute to the post-mitotic state of mammalian CM.

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Adult mammalian CM grow primarily through hypertrophy, which refers to an increase in cell size in the absence of cellular division [7]. In addition, mammalian CM can duplicate their DNA in the early postnatal period without undergoing mitosis and cytokinesis, leading to the formation of mononucleated polyploid or binucleated CM [8–10]. In C57BL/6J inbred mice, a strain commonly used in experimental cardiovascular research, CM binucleation is initiated by a sex-independent mechanism at postnatal day 5 and ceases at day 14 when 65–90% of ventricular CMs are binucleated [11].

Our review gives a summary of the current understanding of the regulation of CM proliferation during development and regeneration. Very little is about known how human CM mature and proliferate. The available evidence indicates that CM from infants have the capacity to proliferate and can respond to regenerative signaling events in comparison to older children and adults [8,12]. Thus, it would be interesting to study the time window of CM proliferation after birth in large animal models, since it may reveal important clinical implications for repair of congenital heart malformations in infants.

Currently, there is no therapy available which directly improves cardiac function post-injury. Dissection of the underlying molecular mechanisms that promote or inhibit CM proliferation in adult mammals and exploring means to enhance this capacity is important. Available therapies interfere with maladaptive neurohormonal changes that accompany the development of chronic heart failure [7,12]. The development of new treatment options to increase heart performance will improve the quality-of-life patients and reduce healthcare costs.

Inhibition of CM proliferation by Rb on E2f-mediated transactivation of cell cycle factors

The pocket protein family of tumor suppressors, Rb and p130, control CM differentiation in many tissues, including the heart [13]. CM-specific Rb knockout (*KO*) mice show no change in heart size, cardiac function, or CM numbers. In contrast, co-deletion of Rb and p130 in mice led to a 3-fold increase in the heart-weight to body-weight ratios and a significant increase in CM numbers. Moreover, hearts of Rb/p130 double knockout (*DKO*) mice have higher levels of E2f1, myelocytomatosis oncogene (Myc) and G1 Cdks Cdk2/4/6 [14,15]. These findings suggest that Rb and p130 have synergistic and overlapping functions in cell cycle inhibition in adult CM, *in vivo*.

The E2f transcription factor family plays a pivotal role in the coordinated expression of genes necessary for cell cycle progression and cell division [15]. Overexpression of E2f2 and E2f4 by adenovirus-mediated gene transfer in neonatal CM induces S-phase entry [16]. Importantly, only overexpression of E2f2 resulted in CM proliferation, whereas ectopic expression of E2f1 and E2f3 induced apoptosis [16,17]. Expression of E2f5 had no effects on cell cycle progression. E2f1-4 alleviate G0 arrest by inducing cyclin A and E [16,17]. Based on these findings, ectopic expression of E2f2 might serve as a strategy to induce CM proliferation after injury. Next, we discuss the regulation of cyclin-Cdk complexes as important downstream targets of the Rb-E2f-pathway.

Regulation of the cell cycle by the Rb-E2f-Cyclin/Cdk signaling pathway

Progression through the cell cycle is tightly modulated by cyclin-dependent protein kinases (Cdks) together with their associated regulatory subunits, cyclins [18]. Cdks that are directly involved in activation of the cell cycle include the G1-phase specific Cdks2/4/6, the S-phase Cdk2, and the M-phase specific Cdks1/7 (Figure 1). At the start of each phase of the cell cycle, varying levels of cyclins influence the activation of Cdks and the subsequent phosphorylation of their target proteins. Initially, when the cell cycle is inactive, the retinoblastoma protein (Rb) binds and inhibits E2f transcription factors to prevent the transactivation of their pro-proliferative target genes [18]. Upon growth factor stimulation, Rb is inactivated by cyclin D/Cdk4/6-dependent phosphorylation in mid G1-phase which leads to the disassociation of E2f from Rb. Free E2fs induce the progression through the cell cycle through transcriptional activation of many cell cycle promoting genes, including cyclin E and cyclin A at the G1/S phase boundary [19]. In late G1-phase, induction of cyclin E-expression by E2f and formation of catalytically active cyclin E/Cdk2 complexes initiate DNA replication and centrosome duplication to prepare for mitotic spindle formation in M-phase [20].

The progression through S- and G2/M-phase of the cell cycle is regulated by cyclin A which activates Cdc6, an important component of the DNA replication machinery that is required at the origin recognition sites in the genome [21]. In addition, Cyclin A/Cdk2 complexes are also involved in G2-phase to prepare the cell for the transition into M-phase. Overexpression of cardiac-specific cyclin A2 in transgenic mice evoked cardiac hypertrophy along with a larger number of smaller CM, implying that ectopic delivery of cyclin A2 contributes to CM proliferation [21]. Another independent study confirmed that ectopic cyclin A2 led to an increase in the degree of CM mitosis along

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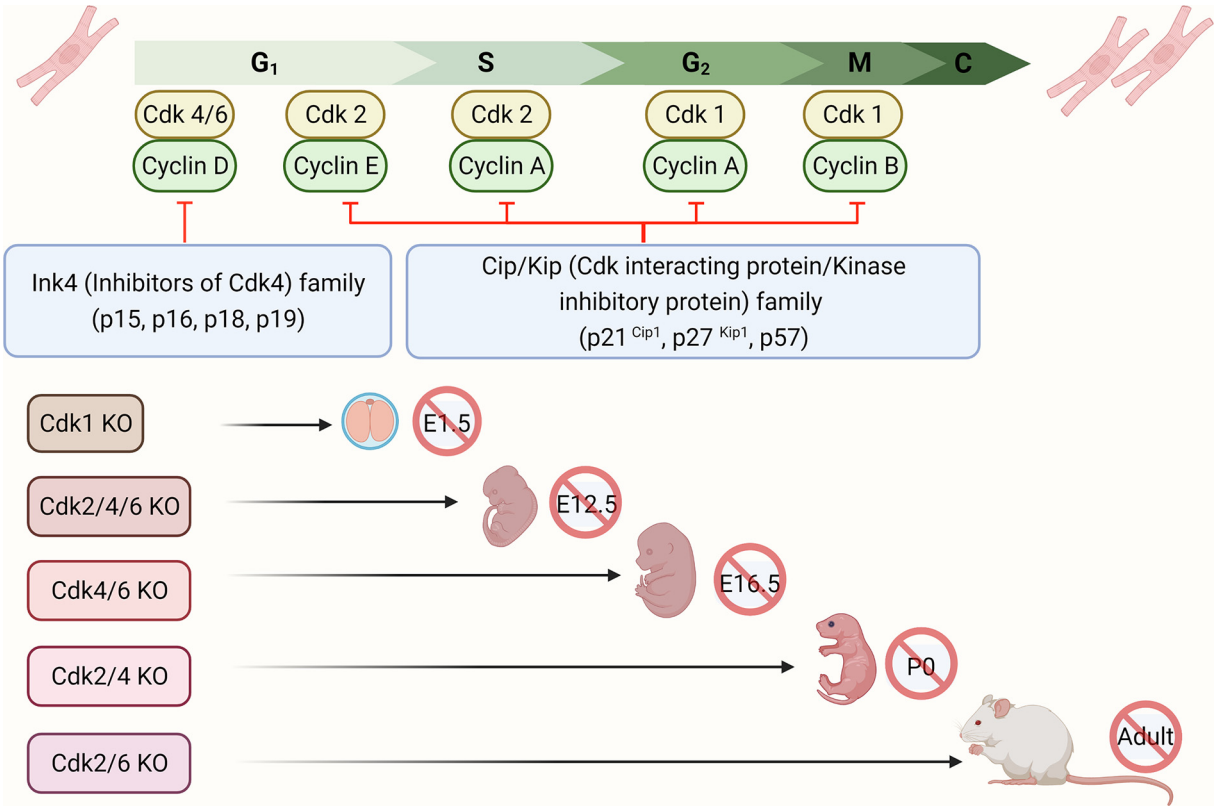

Figure 1. CM cell cycle regulators and inhibitors

The cycle is categorized into distinct phases that follow one another in a timely coordinated manner. During G1-phase, the cell undergoes growth and prepares for DNA and centriole replication that takes place at S-phase. At the S/G2-phase boundary, a cell synthesizes all components of the protein machinery required for cell division during which nuclear division and cytoplasmic division occur. Multiple Cdk-cyclin complexes play specific roles at various stages that monitors and regulates the integrity and progression of a cell during the cell cycle. These complexes include Cdk4/6-cyclin D and Cdk2-cyclin E that are active in G1-phase, Cdk2-cyclin A for S-phase, Cdk1-cyclin A for G2-phase and Cdk1-cyclin B for mitosis. The activity of the Cdk-cyclin complex is controlled by two families of Cdk inhibitors, the Ink4 (Inhibitors of Cdk4) family which modulates Cdk4/6 activity, and the Cip/Kip1 (CDK interacting protein/Kinase inhibitory protein) family which controls the activity of Cdk1/2. Knockout of various Cdk has been analyzed. The stop sign represents the extend of the developmental stage at which the mutated mice strains survived. Mice expressing all Cdks except for Cdk1 did not progress beyond E1.5. On the other hand, mice expressing only Cdk1 (Cdk2/4/6 KO) survived to E12.5. Ablation of Cdk4/6 in mice enabled fetal development to E16.5, whereas Cdk2/4 KO mice died at P0. Mice lacking Cdk2/6 survived to adulthood. Created with BioRender.com

with enhanced cardiac function [22,23]. Intriguingly, a rise in intracellular amounts of cyclin A2 suffices to drive cells all the way through G1/S- and into M-phase and cytokinesis. Presumably, this increase in the abundance of cyclin A2 removes a cellular proliferation block(s) e.g., down-regulation of p27 and upregulation of the E3 ubiquitin ligase Skp2 that allows for cell division to occur. Of course, the involvement of other mechanisms cannot be ruled out.

In mammalian cells, G2/M transition is initiated by cyclin B/Cdk1 which is required for the breakdown of the nuclear double-membrane, chromosome condensation, and activation of the anaphase promoting complex/cyclosome (APC/C) complex [24]. Independent studies showed that adenoviral-mediated overexpression of cyclin B1 in conjunction with Cdk1 in isolated adult rat CM *in vitro* increased Cdk1 kinase activity, chromosome condensation, mitotic histone H3 phosphorylation and efficiently induced CM division [24,25]. In an impressive investigation, the Srivastava group recently demonstrated that simultaneous ectopic expression of Cdk1/4 and cyclin B1/D1 could efficiently stimulate CM proliferation in isolated adult CM from human, mouse and rat *in vitro*, and adult mouse CM *in vivo* [25]. This group employed a lineage tracing system to overexpress all four factors exclusively in CM, *in vivo*. Remarkably, this approach gave rise to continued proliferation in 15–20% of CM which contributed to improved cardiac

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function post-myocardial infarction (MI) [25]. This exciting report demonstrated for the first time, that introduction of a set of defined factors is sufficient to induce ongoing CM proliferation in the adult heart of a small mammal.

Mechanisms of Cdk-dependent control of CM proliferation during G1/S and G2/M-phase transition of the cell cycle

The progression through the mammalian cell cycle requires a timely regulation of cyclin-Cdk activities. For instance, cyclin D1-Cdk4/6 are the dominate complexes in early G1-phase [26]. Later in G1, transition into S-phase and DNA replication is initiated by cyclin E/Cdk2 and followed by cyclin A/Cdk2 at the boundary to G2/M transition. Thus, the association of cyclin B and A with Cdk1 is most critical for the G2/M-phase transition [26]. In contrast, the requirement of Cdk2 in the cell division process has remained unclear since Cdk2 deletion in mouse embryonic fibroblasts (MEF) and other somatic cells *in vitro* failed to significantly impair proliferation [27,28]. In addition, germline ablation of murine Cdk2 led to smaller but viable Cdk2 KO mice that were sterile due defects in meiotic cell division. This surprising result suggests that although Cdk2 might play a role in regulating proliferation in cultured cells, it is not necessary for mitosis during development, *in vivo*.

The functional roles of Cdk2 have also been studied in CM, *in vivo*. Using transgenic mice that overexpress Cdk2 specifically in adult CM, an increase in the expression of Cdk4 as well as cyclin A, D3 and E was observed [29]. Increased Cdk2 levels led to an induction of DNA synthesis, and to an increase in the number of smaller, mononucleated CM indicating enhanced CM division in these mice. Intriguingly, adult mouse hearts overexpressing Cdk2 had normal fractional shortening albeit an activated fetal gene program, including beta-myosin heavy chain (β-MHC) and atrial natriuretic factor (ANF). However, transgenic Cdk2 mice subjected to pressure overload developed pathological cardiac hypertrophic [29]. In this model, no increase was observed in the number of mononuclear CM.

Both Cdk4 and Cdk6 share 71% of amino acid homology and both are able to form complexes with cyclin D that can inactivate Rb through phosphorylation [30]. Despite these similarities, there are also differences between Cdk4 and Cdk6. For example, in murine astrocytes, Cdk4 is predominately present in the nucleus whereas Cdk6 is cytoplasmic [31]. Moreover, Cdk4 inhibits Rb activity by phosphorylation specifically at threonine residue 826, whereas Cdk6 is responsible for Rb inactivation by phosphorylation at the threonine 821 site [32]. While deletion of Cdk4 or Cdk6 results in viable offspring, Cdk4 KO mice tend to be 50% smaller at birth which is not compensated for during the postnatal development [33]. MEF derived from Cdk4 KO can proliferate *in vitro* but these cells showed a delayed transition into S-phase due to p27-mediated inhibition of Cdk2. Intriguingly, p27 can also directly contribute to the activation of cyclin D/Cdk complexes in addition to its inhibitory impact on Cdk2 [33]. Presumably, a competition between binding of p27 to cyclin D/Cdk or cyclin E/Cdk2 is responsible for this effect. Sequestration of p27 by cyclin D/Cdk at the end of G1 enables cyclin E/Cdk2 complexes to be activated and this contributes to S-phase entry [33]. This model can explain, that in the absence of Cdk4, S-phase entry is blocked by p27-dependent inhibition of cyclin E/Cdk2. This view is corroborated by the finding, that MEF deficient for Cdk4 and p27 fail to exhibit a delay in G1/S-phase transition [33].

Deletion of both Cdk4 and Cdk6 results in embryonic lethality at embryonic days E14.5-18.5, presumably due to severe anemia [34]. Cdk4/6 KO exhibited normal organogenesis indicating that most cells of these mice could maintain normal cell division. This finding was corroborated further by analysis of MEF lacking Cdk4/6 which proliferated without any signs of major aberrations including serum-dependency and kinetics of entry into S-phase [34]. These data demonstrate that D-type Cdk4/6 are not required for S-phase entry and that alternative mechanisms must exist to confer cell division in response to growth factor stimulation.

Similar to Cdk4/Cdk6 DKO mice, co-ablation of Cdk2 and Cdk4 led to early embryonic death at day E15 due to abnormal heart morphology [35]. Further *in vitro* studies of Cdk2/4-deficient MEF isolated from this mutant strain revealed that loss of Cdk2/4 impairs the phosphorylation and thus inactivation of Rb which led to lower expression levels of E2f-regulated cell cycle factors Cdk1/2 and cyclin E/A [35]. MEF lacking Cdk2/4 displayed impaired S-phase entry and a lower rate of cell division. Intriguingly, inactivation of p27 in Cdk4/6-deficient MEF could not rescue this proliferative defect. These findings suggest that the combined action of Cdk2/4 is required to activate E2f-dependent expression of S-phase promoting genes through Rb inactivation *in vivo* [35]. In this model, Cdk4 and/or Cdk6 initially phosphorylate Rb during early G1-phase, which leads to its dissociation from E2F contributing to gene expression of Cyclin E and cdk2. Through this positive feedback loop, E2F can further phosphorylate Rb and drive cells past the G1/S restriction point and into S-phase [36].

In response to the loss of Cdk2/4, mice displayed higher levels of cyclin D1/Cdk6 complexes that participated in Rb hyperphosphorylation [35]. Intriguingly, the time of death of Cdk2/4KO mice coincided with the drop in cyclin D1 expression and a concomitant decrease in cyclin D/Cdk complexes. Reportedly, Cdk1 and Cdk6 can partially

compensate for the loss of Cdk4 and Cdk2. Cdk1 and Cdk2 can phosphorylate a broad range of similar *in vitro* substrates including Rb. In fact, recombinant Rb was used to determine that Cdk1 activity in Cdk2/4 DKO mice was at levels similar to wild-type embryos [35]. However, Cdk1 activity was not sufficient to compensate for the loss of Cdk2/4 and the presence of active Rb which caused the repression of E2F-dependent transcription of cell cycle promoting genes. Since E2F-target genes are responsible for entry into S-phase and for cell cycle progression, the down-regulation of this factors can be viewed as the primary cause underlying the proliferation defects in MEF from Cdk4/6KO mice [34].

Single KO mice of Cdk2 or Cdk6 are sterile. Cdk2/6 DKO mice are viable and show normal cellular proliferation [35]. Surprisingly, co-deficiency of Cdk2/4 in mice was embryonic lethal at day E13.5 due cardiac and hematopoietic defects [35]. Embryonic hearts from this strain had thinner ventricular walls that was caused by impaired CM proliferation. However, triple KO MEF lacking Cdk2/4/6, fail to show any perturbations in cell cycle control and cell division *in vitro* [37]. These MEFs maintain normal protein levels of Cdk1 and Cdk7, and mitotic cyclins D1, D2, E1, A2 and B1 in the presence of low p27 expression. Combined, these findings strongly support the view, that the inactivating phosphorylation of Rb through Cdk1 is important for CM proliferation during mid gestation.

Cdk1 is required for proliferation of mammalian cells

There are many functional overlapping functionalities between Cdk1 and Cdk2/4/6. Genetic inactivation of Cdk2/4/6 failed to abrogate early embryonic murine development [37]. These animals continue to express Cdk1 and that allows cell division to occur up to mid gestation. Therefore, it was surprising that embryos lacking a single allele of Cdk1 failed to develop beyond the two-cell embryonic stage. This was a puzzling finding since Cdk1 can compensate for other Cdks in the absence of Cdk2/4/6 including complex formation with cyclins A, B, D and E. However, the disruption of Cdk1 gene function in *in vivo* strongly indicates that Cdk1 exerts unique and non-redundant functions in mammalian proliferation that cannot be provided by Cdk2/4/6 [37]. In addition, replacement of Cdk1 by Cdk2 in the Cdk1 gene locus does not prevent early embryonic death in a mouse knockin model [38]. Again, this important result clearly demonstrates that Cdk2 cannot functionally replace Cdk1.

Many studies were performed in the field of cancer biology to explore the importance of Cdk1 in the regulation of cell division. For instance, inhibition of Cdk1 in human cancer cells lines using small molecule inhibitors revealed an important role of Cdk1 in the prevention of DNA re-replication and induction of cytokinesis after mitotic exit [39]. These studies were extended further employing liver tumor cells and MEFs [40]. Independent investigations reported that Cdk1 can inhibit DNA re-replication and promote M-phase entry by sequestering cyclin A2 away from Cdk2. In the absence of functional Cdk1, MEFs undergo DNA re-replication due to elevated cyclin A-Cdk2 activity and become permanently arrested at the G2/M-phase boundary [40].

Cdk1 is essential for cell viability and its overexpression together with cyclin B1 has been linked to many types of proliferative disorders, including melanoma, lymphoma, and lung cancer [41]. In response to environmental stress, such as nutrient depletion or exposure DNA damaging agents, normal cells can stop cell cycle not only through Cdk2, but also by inhibiting Cdk1 activity through up-regulation of Wee1 and Cdc25 [42]. In contrast, unregulated Cdk1 activity can perturbate the tumor suppressor p53-regulated DNA damage-signal transduction pathway which results in unrestricted cell proliferation [43]. Once the damage is repaired in normal cells, Cdk1 repression is released, thereby allowing the cells to progress through mitosis.

Cdk1 is an attractive drug target in the development of cancer therapies development [43]. Cdk1 can directly regulate the centrosome cycle in mitosis, and overexpression of Cdk1 causes centrosome multiplication in mice lacking functional p53 [44]. In turn, abnormal amplification of centrosomes can cause mitotic defects as well as faulty chromosome separation that are characteristics of many cancers.

The functional role of Cdk inhibitors in the regulation of cell cycle progression

Principally, Cdks are subjected to regulation by Cdk inhibitors (Cdki) of the Cip/Kip and Ink4 family [45]. Ink4 family members inhibit Cdk4 and Cdk6 whereas p21, p27 and p57 inhibit Cdk2 and Cdk1. Here, we will focus on the specific roles of p21 and p27 in the regulation of CM proliferation. It has been well established that the cell cycle block in CM correlates with the loss of cyclin/Cdk expression and a concomitant increase in cell cycle inhibitory p21 and p27 [46–49]. In turn, adenoviral gene delivery of the cell cycle activating E2F1 to cultured rat neonatal and adult CM induced DNA synthesis that mechanistically involved down-regulation of p21 and p27 [50,51]. Transcriptional activation of p21 gene transcription in normal cells is mediated by the tumor suppressor p53 [52]. Surprisingly, p21KO mice are developmentally normal and are not tumor prone throughout adulthood [53]. MEFs isolated from these

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mice can also undergo a partial growth arrest in response to DNA damage albeit with less efficiency [54]. Proliferating cell nuclear antigen (PCNA), an auxiliary protein of DNA polymerases, is involved in DNA replication, repair, and serves as a marker for S-phase in microscopical analyses [55]. Adult CM from p21 KO showed elevated PCNA protein levels but do not contain an increased number of CM. In stark contrast with p21 KO mice, p27 KO animals are born with multiorgan hyperplasia [56]. Mechanistically, antiproliferative signals stabilize p27 by inhibiting its proteasomal degradation through posttranslational modifications, thereby enabling p27 to inhibit proliferation [57–59]. Combined, these findings have established that p27 is a potent inhibitor of cell growth and division.

To understand the importance of p21 and p27 for Cdk2 regulation *in vivo*, p21/p27 DKO mice were generated [60]. Organs and MEFs of these DKO mice had abnormalities in cell cycle regulation similar to single KO mice of p21 and p27. These data suggest that p21/p27 can have other targets than Cdk2, in particular Cdk1. In favor of this view, mice lacking p27 or p27/Cdk2 showed higher S- and M-phase activity [61]. p27/Cdk2 DKO also contained cyclin E1-associated kinase activity, which under normal conditions is dependent on its association with Cdk2. However, in these mice cyclin E1 is associated with Cdk1 presumably explaining why cell cycling can be maintained in this strain.

An elegant study by Hauck et al. found out that p27 binds to and regulates Casein kinase 2 alpha prime (CK2α') in isolated neonatal CM *in vitro* and in adult mouse CM, *in vivo* [59]. These data combined with previous results showing that p27 KO mice develop cardiac hyperplasia underscore the importance of p27 in the regulation of CM proliferation. The cardiac growth factor angiotensin II (Ang-II), triggers proteasomal p27 degradation through CK2α'-mediated phosphorylation of p27 [59]. Conversely, p27 can inhibit CK2α' in the absence of Ang-II. Thus, p27 expression in post-mitotic CM is controlled by extracellular growth factor signaling as it occurs also in proliferating cells. In addition, hearts from p27 KO mice exhibit a stronger hypertrophic growth in response to pressure overload. All these findings confirm that p27 inhibition by CK2- CK2α' is important for agonist- and stress-induced hypertrophy [59]. Combined, the interaction between p27 and CK2α' form a regulatory feedback loop in postmitotic CM that is like the feedback loop of p27 with Cdk2 that controls cell proliferation.

Regulation of CM nucleation during the prenatal and postnatal period

The degree of CM proliferation in the murine heart shows significant differences during embryonic and fetal development. The four-chambered mammalian mouse heart is formed at embryonic day E12.5 [62,63]. Proliferation rates after E11 decrease, but still reach the number of 1 million CM per heart by day E17.5. Taken together, CM proliferation in mice peaks around E10-12 and then decreases until birth.

Genesis of mammalian CM during normal aging occurs at a low rate [64]. Mechanistically, formation of new CM in adult rodent and human hearts can principally occur by proliferation of endogenous CM or cardiac stem cells [65]. Reportedly, the annual proliferation rate of adult mouse and human CM *in vivo* is less than 1%. Two studies identified existing CM as the dominant source of CM replacement during murine heart homeostasis and post-MI [65,66]. Under these rather dissimilar conditions, cell cycle activation gives rise to new diploid mononucleate CM as well as polyploid and multinucleated CM. In contrast, the contribution of resident stem/progenitor cells to adult CM homeostasis is very low at baseline and post-MI [67–69].

Analysis of genomic C^{14} concentrations demonstrates, that 74% of CM were mononucleated, 26% were binucleated, and 1% were trinucleated [70]. This ratio remains unaltered during lifetime. Moreover, the final CM number is reached one month after birth and does not change over the lifetime. This result, however, could not be confirmed. In an independent investigation a 3.4-fold increase in CM number was found between the age of 1 and 20 years [71]. The study by Bergmann et al. employed a CM-specific nuclear marker in their analysis, suggesting a more accurate identification and therefore, quantification of CM [70]. Moreover, adult human CM show a low mitotic index *in vivo*, which is in good agreement with a low turnover rate [71]. Replacement of human CM takes place at a rate of 2% per year during the first 10 years of life. After that, replacement decreases to 1% per year by 70 years.

Human CM nuclei are mostly diploid during the first years of life [70]. During the second decade after birth, the DNA content per CM nucleus increases 1.7-fold. This study demonstrates that the postnatal CM turnover is highest with around 7% at 8 years and declines thereafter to 0.8% per year at the age of 20 [70]. Thus, the majority of CM in the human heart is generated in the perinatal period.

Although CM renewal has been observed at a rate of 1% among young adults, and less than 0.5% among the elderly, the rate of cell turnover is insufficient to 'repair' the heart after injury [70]. The main methodological challenge in determining CM proliferation is the assay used in this study that does not discriminate between CM division and multinucleation or polyploidization. Approximately 80% of the entire CM population in the left LV will not be replaced during lifetime [70]. In a 75 years-old individual, 39% of CM have been produced postnatally, and of this

population, 36% were generated by 10 years of age. CM turnover in the RV is not significant different compared with the LV with 50% of CM being renewed at 75 years of age [70].

In mouse and rat CM, binucleation is initiated between postnatal day 4–6. At neonatal day 14, 65% of those CM are binucleated [62,72,73]. Binucleation may contribute to CM survival during periods of stress [74]. Alternatively, binucleation could occur to meet the high metabolic demand of the rapidly growing heart during adolescent and young adulthood [75]. According to a recent study, a proliferative burst of mouse CM at 15 days post-birth gives rise to binucleated CM [76]. This replicative event is mediated by the IGF-1-Akt-thyroid hormone axis. However, several independent studies were unable to confirm a preadolescent burst in CM proliferation [72,73].

The adult murine and human heart lacks an appreciable amount to regenerate after injury. This result is in stark contrast with the situation in neonatal mouse hearts at 1 day post-birth [77], salamanders [78–80] and zebrafish [81,82] that can mount a regenerative response based on CM proliferation.

Tissue regeneration shows a divergence across phylogeny and ontogeny, but the underlying mechanisms remain unknown. Loss of mammalian CM regenerative potential correlates with cell-cycle arrest and polyploidization and development of endothermy early after birth [83]. Inactivation of thyroid hormone signaling decreases mouse CM polyploidization, cell cycle exit and maintains regenerative potential into adulthood. Strikingly, exogenous thyroid hormones can inhibit heart regeneration zebrafish [83]. Thus, loss of adult CM proliferation in mammals appears to be triggered though up-regulated thyroid levels perinatally. This could represent a trade-off for physiological advantages that are associated with acquired endothermy.

The majority of CM in non-regenerative hearts are polyploid [84], whereas regenerative hearts contain mostly diploid CM [85,86]. Further studies provided additional experimental evidence for a negative correlation between the DNA content of CM and regenerative capacity [87,88]. Gonzalez et al. employed a transgenic zebrafish model to express a dominant-negative mutant version of Epithelial Cell Transforming 2 (dnEct2) [89]. Ect2 is a guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases (RhoGEF) that is required for cytokinesis in animals and is up-regulated in dividing CM. Intriguingly, overexpression of dnEct2 *in CM in vivo* led to an increase in ploidy. Hearts from this transgenic mouse strain showed scar formation in a neonatal apex resection model. In contrast, control hearts were able to regenerate the resected region without scar formation due to CM proliferation [89]. These data imply that the loss of functional Ect2 inhibits CM proliferation post-injury leading to CM polyploidy.

Recent reports suggested that mononucleated CM are more sensitive to mitogenic stimuli in comparison with binucleated CM [90]. CM binucleation was increased in Ect2-deficient CM and accompanied by decreases in E2f-traget gene expression. Thus, the Rb-E2f pathway can regulate the generation of these two CM populations during the early neonatal period and their maintenance in adult CM. Moreover, perinatal binucleation inhibited CM proliferation and cardiac repair in a regeneration model in neonatal mice [90]. While Rb is required for the inhibition of E2f-dependent gene transcription, it is not involved in the initiation of binucleation.

There are still large gaps in our understanding of the mechanisms that govern CM binucleation. For example, the closely related mouse inbred strains BALB/cJ and BALB/cByJ show a substantial variation in their levels of mononucleated adult CM (6.6% versus 14.3%) [91]. In these strains, non-epigenetic mechanisms such as certain polymorphisms located in X-linked and autosomal genes can influence the outcome of mitosis and karyokinesis in a CM-nonautonomous fashion.

During the progression from prenatal to postnatal development, an important shift occurs in the regulation of the cell cycle. This change includes the expression of Cdks, cyclins, and Cdkis and are reflective of a cell cycle exit [92–98]. Shortly after birth, mammalian CM cease to proliferate with the commitment downregulation of cell cycle promoting genes, such as Cdks1, 2, 4 and cyclins D1, E, A, B1, E2F1-3 and the E3 ubiquitin ligase Skp2.

In mice and rats, a burst of cyclin/Cdk activity is observed around postnatal day 5 and corresponds to CM binucleation [47,92]. By postnatal day 14 the expression levels of cyclin/CDKs are reduced and CM binucleation is completed. In mice, complete inhibition of Cdk activities by Cdkis occurs at postnatal day 14 [47]. Interestingly, there is a strong reciprocal correlation between the down-regulation of cyclin E/A-Cdk and the up-regulation p21 and p27 at 5d post-birth [92]. The number of mononuclear CM in adult mouse inbred strains is highly variable (up to 7-fold) [90,99]. In C57BL/6 mice, commonly employed for cardiological studies, 8–10% of CM are mononucleated.

Intriguingly, triple KO mice of cyclin D1, 2 and 3 develop hypoplastic ventricular walls and severe anemia and die in mid/late gestation at embryonic day E16.5 [100]. In contrast, D-type cyclin deficient MEFs can proliferate normally but exhibit an increased dependency on growth factors for G1/S-phase entry. Proliferation of these MEFs is insensitive to p16ink4a and requires Cdk2 [100]. These data suggest the existence of alternative mechanisms that acts independently from D-type cyclins in the stimulation of cell cycle progression.

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In good agreement with these findings, D-types cyclins are also critical regulatory factor in the initiation of DNA replication in CM [101]. For example, overexpression of cyclin D1 in murine CM induced DNA replication and multi-nucleation without cell division [86]. Moreover, overexpression of cyclin D2 led to CM proliferation and improvement in cardiac function following cardiac injury [102].

Cell cycle withdrawal via Cdki-based inhibition and Foxo1/3 transcription factors

The Forkhead O (Foxo) family of transcription factors exerts important functions in CM proliferation and growth through the regulation of Cdkis p21 and p27 [103]. Germ line deficiency f Foxo1 KO evokes early embryonic death prior to cardiac morphogenesis [104]. Foxo3 KO mice are born without an apparent phenotype but develop cardiac hypertrophy and heart failure later on in adult life [105]. While Foxo1 and Foxo3 are both expressed during embryonic heart development, only Foxo3 is detectable in the heart after birth [103]. In CMs, unphosphorylated Foxo1 is localized to the nucleus between embryonic day E18.5 up to postnatal day 7, coinciding with the initiation of CM cell cycle withdrawal around the perinatal period. Isolated embryonic CM proliferation is stimulated by growth factor-dependent activation of the Pi3K/Akt signal transduction pathway. Mechanistically, Pi3K/Akt signaling events phosphorylate Foxo1 at serine 256, causing its exportation from the nucleus [106]. With the translocation of Foxo1 out of the nucleus, the levels of p21 and p27 decrease, leading to CM proliferation [103].

In contrast, overexpression of a dominant-negative mutant version of Foxo1 leads to embryonic lethality at day E10.5 coinciding with early activation of Cdki and decreased CM proliferation [103]. In good agreement with this finding, murine CM expressing dominant negative Foxo1 displayed increased CM proliferation and decreased Cdki expression at embryonic day E18.5 [103]. Combined, all these observations support the notion that the Foxo1/3-mediated regulation of Cdkis is important for the regulation of CM cell cycle exit in the early neonatal period.

Wnt/β-catenin signaling is important for mammalian CM proliferation and cardiac repair

Wnt signalling proteins have multiple regulatory roles including development, proliferation, survival, migration and adhesion [107]. Upon interaction with a complex of receptor proteins including lipoprotein-related receptor protein (LRP5) and LRP6 along with the Frizzled family, Wnt ligands can activate this canonical signaling pathway (Figure 2) [107]. In the inactive state, the Wnt downstream effector Ctnnb1 (β-catenin) becomes phosphorylated by casein kinase-1 (CK1) and glycogen synthase kinase-3 (Gsk-3β), leading to the degradation of Ctnnb1 in the cytoplasm. Activation of the Wnt receptor, however, induces the translocation of the Ctnnb1 degradation complex to the plasma membrane, where it becomes inactive [107]. Subsequently, the concentration of Ctnnb1 at the cytoplasm increases which triggers cytoplasmic Ctnnb1 to be transported into the nucleus. Nuclear Ctnnb1 complexes with transcription factors of the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) and induces the transcription of Wnt target genes including various cell cycle genes.

Adult zebrafish hearts can replace CM lost after injury [108]. CM regeneration in zebrafish occurs through a mechanism that employs dedifferentiation and proliferation of differentiated CM. Bertozzi et al. reported, that active Wnt-Ctnnb1 signaling is required for autonomous CM proliferation and dedifferentiation, and for scar maturation after apical resection [108]. However, these results are in conflict with a previous study that supports an inhibitory function for Wnt-Ctnnb1 signaling in the cardiac regenerative response in zebrafish [109].

Dishevelled-1 (Dvl-1) is a positive regulator of the Wnt pathway [110,111]. Increases in Dvl-1 protein expression in CM was observed after trans-aortic banding in mice suggesting that Dvl-1 can have a functional role in the development of pathological hypertrophy in response to hemodynamic stress. To examine the potential role of Wnt signaling on the postnatal heart, CM-specific Dvl-1 was overexpressed in a transgenic mice model. At 3 months of age, Dvl-1-dependent activation of Wnt signaling induced severe cardiomyopathy with excessive remodeling [110,111]. Notably, treatment of isolated rat CM with siRNAs to Dvl-1 inhibited hypertrophic growth in response to β-adrenergic stimulation suggesting that Wnt signaling is critical for the proper regulation of cardiac hypertrophy.

Another important protein involved in the Wnt signaling pathway is Gsk-3β. Reportedly, CM-specific Gsk-3β KO in adult mice did not reduce infarct size post-MI [112]. However, loss of Gsk-3β improved cardiac function at 8 weeks after MI due to CM proliferation in the border zone. In contrast, smaller infarcts were observed in Gsk-3β KO mice subjected to ischemia–reperfusion injury [113]. Inhibition of Gsk-3β signaling by the small pharmacological inhibitor 6-bromoindirubin-3′-oxime (BIO) led to proliferation of isolated neonatal rat CM [114]. Another Gsk-3β

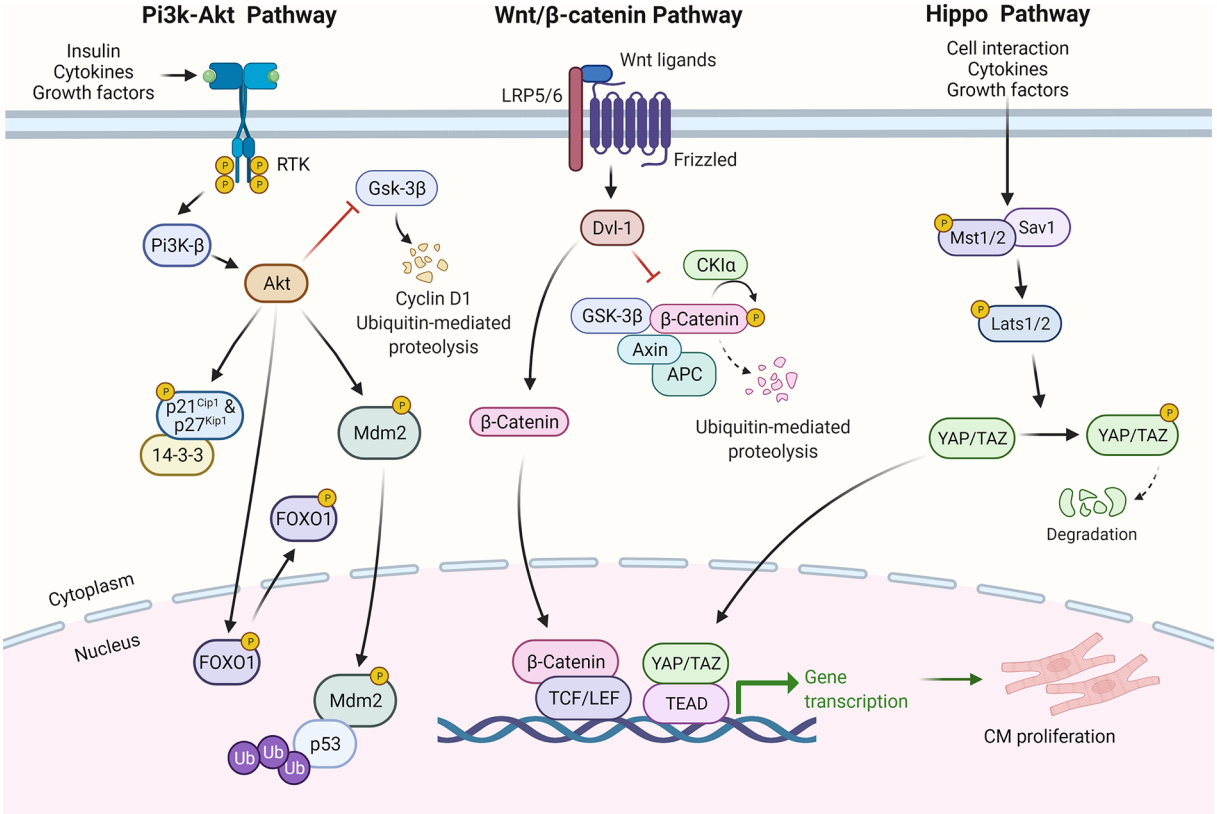

Figure 2. Signaling pathways associated with CM proliferation

The Pi3K-Akt signal transduction pathway becomes activated by a range of extracellular signals such as insulin, cytokines and growth factors that act on ligand-specific receptor tyrosine kinases (RTK). Upon ligand binding, RTKs form a dimer and autophosphorylate each monomer for activation. Protein kinase B (Akt) becomes fully active when threonine 308 and serine 473 residues are phosphorylated by phosphatidylinositol-3 kinase (Pi3K-β). Activated Akt controls proliferation through regulation of the p53/Mdm2 tumor suppressor pathway. Phosphorylation of Mdm2 by Akt leads to its translocation to the nucleus. In the nucleus Mdm2 ubiquitinates the transcription factor p53 thereby promoting its proteasomal degradation and down-regulation of p21, a p53 target gene. Active Pi3K-Akt pathway signaling phosphorylates nuclear Forkhead O (Foxo) family of transcription factor that leads to its inactivation by cytoplasmic translocation. Pi3K-Akt pathway facilitates G1/S-phase progression by blocking p21 and p27 via phosphorylation. Once phosphorylated, the cell-cycle inhibitors adopt a cytosolic localization mediated through binding to 14–3-3 proteins. Constitutive expression of Akt inactivates Gsk-3β by phosphorylating it at Serine 9, leading to prolonged stabilization of cyclin D1 protein. Gsk-3β also has a role in Wnt/β-catenin signal transduction pathway where it promotes degradation of β-catenin (Ctnnb1). When the Wnt/β-catenin pathway is inactive, β-catenin is phosphorylated by the combined action of casein kinase-1 (CK1) and Gsk3-β. This phosphorylation leads to cytosolic β-catenin degradation by Axin, and adenomatosis polyposis coli (APC). Wnt ligand activates a canonical signalling pathway upon interaction with Lrp5 and Lrp6 along with the Frizzled family. Active Wnt receptor recruits dishevelled (Dvl)-1 to the plasma member where it provides a platform for Axin and Gsk3-β binding, which in turn, inhibits their activity. This causes a rise in cytoplasmic β-catenin levels and its uptake in the nuclear compartment. Nuclear β-catenin can interact with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) to induce the transcription of cell cycle promoting genes. Upon activation of the Hippo pathway the scaffold protein Sav1 interacts with and enhances the activity of Mst1/2 (mammalian Ste20-like kinases 1/2). The Mst1/2-Sav1 complex activates the Lats1/2 (large tumor suppressor 1/2) which, in turn, phosphorylate YAP (Yes-associated protein) causing its degradation in the cytoplasm. In the case of inactive Hippo pathway, the unphosphorylated YAP forms a complex with TAZ and TEAD in the nucleus, and along with other transcriptional factors they induce transcription of genes involved in cell cycle progression. Created with BioRender.com

inhibitor, CHIR-99021, can promote proliferation through canonical Wnt signalling in isolated human neonatal atrial CM [115]. All these findings support an important role for Gsk-3β as a mediator of activated Wnt signaling in the normal heart.

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The peroxisome proliferator-activated receptor delta (PPARδ) is involved in the development of cancers and other proliferative disorders [116]. The PPARδ gene encodes nuclear hormone receptor and transcription factor for many genes involved in fatty acid oxidation, the main energy source of the normal adult mammalian heart [117]. It has been very well established, that adult Zebrafish can regenerate the heart after apical resection [88,108]. In this regard, inhibition of PPARδ in zebrafish reduces CM proliferation during heart regeneration [117]. In mice, PPARδ KO down-regulated myocardial fatty acid oxidation (FAO) and led to hypertrophy and cardiomyopathy [118]. In contrast, activation of PPARδ via carbacyclin induces CM proliferation, *in vitro*. Furthermore, activation of PPARδ in mice induced with MI stimulates CM proliferation and gradually improved cardiac function over 6 months post-MI [117].

The loss of regenerative capacity of the heart after birth coincides with the switch from anaerobic glycolysis to FAO and mitochondrial (mt-) oxidative phosphorylation to generate ATP [119]. Reactive oxygen species (ROS), byproducts of mt-OxPhos production, can block proliferation through activation of the p53-dependend DNA-damage response (DDR) pathway [120].

A recent study analyzed the exciting possibility that FAO inhibition could trigger CM proliferation in the adult mouse heart [121]. When the authors fed fat-free milk to block FAO in neonatal mice, they observed significantly prolonged CM division over the first week of life. Next, the group generated an inducible, CM-specific pyruvate dehydrogenase kinase 4 (PDK4) knockout mouse model. In hearts of these mice, metabolization of pyruvate through mt-OxPhos was inhibited, whereas glycolytic oxidation of pyruvate was elevated. This genetic manipulation effectively increased glycolysis relative to FAO and led to an induction of CM proliferation and improved cardiac function post-MI [121]. Future studies are necessary to determine, whether induction of CM proliferation by FAO inhibition can serve as a regenerative platform to treat heart failure in patients.

The co-existence of polyploid mono- and binucleated CM in adult mammalian hearts suggests that both cell types lost the capacity to correctly undergo mitosis and cytokinesis, respectively. Thus, more knowledge is necessary before cardiac regenerative therapies can be utilized for the treatment of human heart failure. To fill this gap, Pettinato et al. observed in CM from human induced pluripotent stem cell (CM-hiPSC) that p53 can induce polyploidization of CM-hiPSC through downregulation of cyclin B1 [122]. Moreover, engrafting troponin 1-deficient CM-hiPSC in adult rat hearts post-MI markedly increased CM proliferation and cardiac remuscularization *in vivo*. In conclusion, sarcomere organization can inhibit CM proliferation through the p53-DNA damage response pathway. Targeted removal of saromeric proteins to coax CM into proper cell division could be employed as CM replacement strategy to treat MI or congenital heart disease in patients.

Another approach may be through the muscle pyruvate kinase 2 (Pkm2) which catalyzes the rate limiting step for the production of pyruvate in the glycolytic pathway [123]. Given the important role of Pkm2 in the regulation of cancer cell proliferation, a recent study investigated whether CM proliferation can be regulated through Pkm2 [124]. When the Wnt/Ctnnb1 pathway is inactive, CK1 and Gsk-3β phosphorylate Ctnnb1, leading to its proteasomal degradation (Figure 3) [125]. Activation of Wnt-signaling prevents Gsk-3β-dependent Ctnnb1 phosphorylation, which promotes the stabilization and translocation of Ctnnb1 to the nucleus where it can activate genes necessary for cell cycle re-entry including Myc and Cyclin D1 [125]. Cardiac-specific deletion of Pkm2 in adult mice induced CM proliferation with improved heart function post-MI. These effects were abrogated by genetic co-deletion of Pkm2 and Ctnnb1. Mechanistically, Pkm2 interacts with Ctnnb1 in the cytoplasm of CM, where it inhibits Akt-mediated Ctnnb1 phosphorylation at serine 552 and tyrosine 333 [124]. Collectively, this work supports a novel antiproliferative function for Pkm2 in CM (Figure 3). Reducing cardiac Pkm2 expression appears as a useful strategy to treat heart failure in patients post-MI.

In contrast, another investigation found conflicting results regarding Pkm2's role in the regulation of CM regulation *in vivo* [126]. This work showed that Pkm2 was present in CM in the fetal heart and in the early postnatal period but not during adulthood. Ablation of Pkm2 in fetal CM *in vivo* inhibited CM proliferation whereas CM-specific overexpression employing a modified Pkm2-endcoing RNA induced adult CM proliferation post-MI accompanied by an improved cardiac function [126]. From a mechanistic view, ectopic Pkm2 induced nuclear translocation of Pkm2/Ctnnb1 complexes and activation of Ctnnb1-target genes. The major distinction between the two studies is the application of a single injection of modified Pkm2 mRNA into the heart, as opposed to permanent genetic cardiac deletion of Pkm2 in a mouse KO model. Intriguingly, both experimental strategies led to an induction of CM proliferation post-MI with improved heart function.

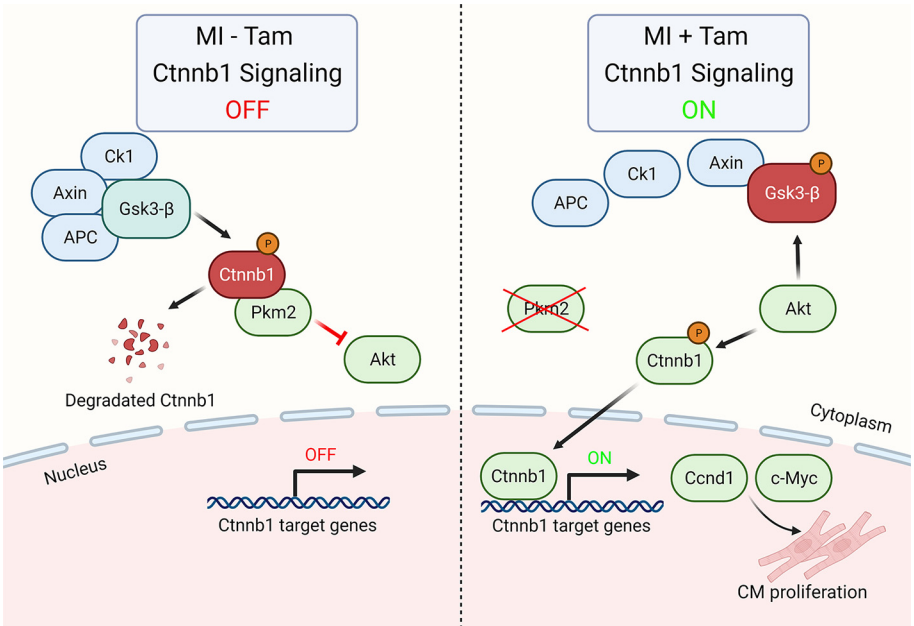

Figure 3. Inhibition of the Pkm2/Ctnnb1 promotes CM proliferation post MI

Model for the regulation of adult CM proliferation by the Pi3K/Akt signal transduction pathway and the Pkm2/Ctnnb1 axis after myocardial infarction (MI) in the absence of Tamoxifen (+Tam; left) and absence (-Tam; right) of Pkm2. In the presence of Pkm2, the inactive Wnt/Ctnnb1 pathway promotes proteasomal degradation of Ctnnb1. This is achieved by the Gsk-3β, APC, Axin and Ck1 phosphorylation of Ctnnb1, as well as Pkm2's ability to sequester Ctnnb1 in the cytoplasm. In the absence of Pkm2, Akt signaling activates the cell cycle through phosphorylation and inactivation of Gsk-3β. The inactive Gsk-3β cannot form a complex with APC, CK1 and Axin, to cause proteasomal degradation of Ctnnb1. Furthermore, Akt phosphorylates Ctnnb1 at serine 552 and tyrosine 333, leading to its nucleus translocation where it promotes transcriptional activation of CM proliferation-associated target genes such as Ccnd1 and Myc. Created with BioRender.com

The Hippo/Yap signaling module is an important for transcriptional regulation of CM proliferation and heart size

The extracellular Hippo signaling transduction pathway is involved in the regulation of cell growth and organ size [127]. The components of this pathway include a series of kinase cascades, namely macrophage stimulating (Mst1/2) and large tumor suppressor kinase (Lats1/2) which ultimately influence the activity two transcriptional coactivators, Yes1 associated transcriptional regulator (Yap) and WW domain containing transcription regulator 1 (Taz) [128]. The protein expression of Yap/Taz as well as their location in the cell can influence proliferation. In the case of inactive Hippo pathway, hypophosphorylated Yap/Taz forms a complex with TEA domain transcription factor 1 (Tead) in the nucleus. This Yap/Taz/Tead complex in conjunction with other transcriptional factors induce transcription of genes involved in cell cycle progression. Conversely, when the Hippo pathway is active, Mst1/2 and Lats1/2 can phosphorylate Yap/Taz and this event causes the degradation of Yap/Taz in the cytoplasm [128].

It has been well established, that the Hippo pathway is a crucial regulator of CM proliferation in the control of heart size [129]. As mentioned above, Yap is a transcriptional cofactor and an important effector of Hippo signaling in the mammalian heart. During embryonic development, Yap activates CM proliferation and regulates heart size through insulin-like growth factor (Igf-1) that regulates the Wnt signaling transduction pathway [130,131]. Genetic deletion of Yap in mouse embryos inhibited CM proliferation causing myocardial hypoplasia and early death at day E10.5. Overexpression of constitutively active mutant Yap increased CM number and heart size through the Igf-mediated Gsk-3β inactivation and expression of Ctnnb1 target genes [130,131].

Conditional KO of core genes of the Hippo pathway such as Mst1/2 and Lats1/2 resulted in massive cardiac overgrowth [132]. In contrast, cardiac-specific deletion of the Yap gene in mice caused decreased CM proliferation and severely thinned myocardial layers that are embryonically lethal [133]. Conversely, embryonic Yap activation increased CM proliferation demonstrating that the Hippo-deficient CM are able to re-enter the cell cycle and complete cytokinesis. Furthermore, adult CM with a suppressed Hippo pathway showed greater proliferation post-MI and in a

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neonatal apical resection models [reviewed in [134]]. Together, all these data indicate that the Hippo pathway inhibits Wnt signalling in developing hearts to control heart size and growth.

The Hippo signaling pathway exhibits crosstalk with many other pathways including Wnt/Ctnnb1, bone morphogenetic proteins (BMPs), transforming growth factor beta (Tgf-β1/2), notch receptors (Notch), G-protein-coupled receptors (GPCR) and Pi3K-Akt signaling pathways [129]. Disruption of this pathway in mice through embryonic deletion of Salvador family WW domain containing (Sav1), one of its key components, increases prenatal CM proliferation, enlargement of the heart, and the development of cardiomegaly at birth [135]. Likewise, for instance, the cardiac KO of Sav1 in mice showed up-regulation of Wnt target genes such as SRY-box transcription factor (Sox2), snail family transcriptional repressor (Snai2) and cell survival-related genes baculoviral IAP repeat containing (Birc2/5). Furthermore, Sav1 KO mice had increased levels of nuclear Ctnnb1 expression indicating activated cardiac Wnt signaling [135].

In addition, CM-specific KO of Sav1 can revert established heart failure in mice post-MI through the activation of parkin RBR E3 ubiquitin protein ligase (Park2) [136]. Cardiac repair by Park2 induced in the absence of Sav1 does not involve resolution of fibrosis but Park2 directed mitochondrial quality control. Since the Hippo pathway is active in ischemic heart failure in humans, inhibition of Hippo signaling could exert beneficial effects in heart failure patients.

Endogenous CM have regenerative capacity at birth, but this capacity is lost postnatally, with subsequent organ growth occurring exclusively through CM hypertrophy [133]. The dystrophin–glycoprotein complex (Dgc) is a large transmembrane complex that links the actin cytoskeleton to the extracellular matrix (ECM) [134]. Dgc deficiency in human patients results in muscular dystrophy implying that it is an essential gene product for cardiac homeostasis. Reportedly, the Dgc component dystroglycan (Dag1) can directly bind to Yap to inhibit CM proliferation in mice. Hippo-deficient postnatal mouse hearts show CM proliferation and cardiac repair without hypertrophic growth at 21 days after apex resection [137]. In contrast, Hippo/DGC DKO mice developed CM hyperproliferation and cardiac hypertrophy after injury. Moreover, Hippo/DGC DKO mice exhibited CM proliferation that participated in cardiac-protection in a pressure-induced heart failure model [137].

Pitx2 and its role in cardiac regeneration

Oxidative stress is caused by an imbalance between ROS production and the ability of an organism to eliminate these toxic intermediates [138]. The heart with its high metabolic FAO rate and limited capacity for regeneration is particularly sensitive to oxidative stress [139,140]. Upon exposure to ROS after MI, the heart undergoes hypertrophic growth with myofibrillar disarray, and re-expression of fetal genes [141]. During the postnatal transition from glycolytic to oxidative metabolism, myocardial ROS is elevated and contributes to the inhibition of CM proliferation [142]. Paired-like homeodomain transcription factor (Pitx2) is induced after apical resection in Hippo-deficient CM and Pitx2-deficient neonatal mouse hearts failed to repair itself. Tao et al. could show that in neonatal hearts Pitx2 promotes regeneration by inhibiting ROS [142]. Cardiac injury through apex resection induces nuclear Pitx2 expression through the key antioxidative transcription factor Nrf2 [143–145]. In turn, Pitx2 activates antioxidants thereby protecting CM and electron transport chain components from oxidative damage [142]. In contrast, adult mouse CM expressing a gain of-function mutant version of Pitx2 could efficiently repair their hearts post-MI. Genomic analysis revealed that Pitx2 activated genes include factors of mt-OxPhos and ROS scavengers. Notably, a subset of Pitx2 target genes was cooperatively regulated by YAP, an effector of the Hippo pathway [142]. Collectively, all these data suggest that Pitx2-facilitated protection against ROS-protective is important for the CM response to injury.

The PI3K-AKT- Gsk-3β pathway is required for Yap- and Ctnnb1-mediated CM proliferation

Protein kinase B (Akt) is a mediator of the Pi3K signaling pathway which regulates CM proliferation, survival, and physiological hypertrophy [146]. Akt becomes fully activated when both threonine 308 as well as serine 473 residues are phosphorylated. Once active, hyperphosphorylated Akt can control proliferation through the regulation of the cell cycle. For instance, phosphorylation of Mdm2 by Akt leads to its translocation to the nucleus [147]. In the nucleus, Mdm2 can promote degradation of p53, which ultimately leads to the reduction of p21 mRNA transcription and release from p21-dependent cell cycle inhibition. The Pi3K-Akt pathway can also reduce the transcription of the Cdki p27 by phosphorylating the transcription factor Foxo1. Akt-dependent Foxo1 phosphorylation retains Foxo1 in the cytoplasm thereby inhibiting Foxo1-mediated transactivation of the p27 gene locus [148]. Combined, Pi3K-Akt signaling facilitates G1/S-phase progression by blocking p21 and p27 via phosphorylation. Once phosphorylated, the cell-cycle inhibitors adopt a cytosolic localization mediated through 14–3-3 binding [147]. However, the Pi3k-Akt pathway can also assist the progression of the cell cycle through other means. For example, constitutive expression of

Akt inactivates Gsk-3β by phosphorylating it at Serine 9 site, leading to prolonged stabilization of cyclin D1 protein [149]. Moreover, Akt can phosphorylate S-phase specific Cdk2 at threonine 39 that enhances the interaction of Cdk2 with cyclin A [150].

Studies in CM have shown that nuclear overexpression of Akt does not alter cardiac size or shape but instead gave rise to an increase in number of smaller CM [151]. The Akt-overexpressing hearts also had enhanced systolic and diastolic function along with improved contractility. Furthermore, cardiac-specific expression of nuclear Akt in transgenics increased CM cell cycling and expanded the cardiac progenitor cell population [152]. Ablation of Gsk-3β, an important Akt target, promoted CM hyperproliferation. This effect was associated with increased expression of the cardiac-specific transcription factor GATA binding protein (Gata4), cyclin D1 and pro-proliferative Myc [153]. Mechanistically, these data support the notion that Pi3K/Akt signaling pathway contributes to the induction of CM proliferation.

In a genome-wide screen based on chromatin-immunoprecipitations combined with DNA sequencing, Lin et al. identified the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (Pik3cb, also called p110β), a catalytic subunit of Pi3K as an effector of Yap [154]. PiK3cb promotes proliferation of isolated neonatal rat CM *in vitro*. Since the PiK3cb gene locus is directly transcriptionally activated by Yap, adenoviral-mediated overexpression of Pik3cb-induced proliferation in isolated CM. In contrast, siRNA mediated Pik3cb down-regulation reduced the mitogenic impact of Yap on CM division [154]. Combined, Pik3cb is a direct YAP target for the activation of the Pi3k-Akt pathway and thus the induction of CM proliferation.

Regulation of CM division and cardiac repair by the neuregulin1-Erbb2/4 signaling pathway

Administration of recombinant Neuregulin-1 (Nrg1) has been shown to induce CM proliferation, cardiac repair and improved heart function in mice subjected to MI [87]. These exciting results suggest that protagonistic activation of Nrg1/erb-b2 receptor tyrosine kinase (Erbb4) signaling may provide a novel clinical for the treatment of heart disease post-MI.

It is well known that heterodimerization of the Nrg1 receptors Erbb2 with other Erbb receptors is required for neuregulin signaling [155]. For example, ErbB2 is essential for CM proliferation at embryonic and neonatal stages whereas it is dramatically down-regulated during the first 2 weeks after birth [156]. Erbb2-deficient mice die before embryonic day E11 due to lack of cardiac trabeculation. Thus, the Nrg1 receptors Erbb2/4 play a pivotal role for heart development and trabeculation.

Nrg1-triggered CM proliferation drops at 7d post-birth due to loss of Erbb2 expression [157]. Moreover, CM-specific Erbb2 knockout revealed that Erbb2 is required for CM proliferation during embryonic and early postnatal development. Overexpression of a constitutively active mutant version of Erbb2 (caErbb2) in neonatal and adult CM resulted in pathological cardiac hypertrophy mediated through the Akt-Erk1/2 and Gsk-3β/Ctnnb1 signal transduction pathways. In contrast, transient overexpression of caErbb2 induced CM dedifferentiation and proliferation with improved heart function post-MI in a manner similar to zebrafish [157]. Thus, Erbb2 can reactivate postnatal CM proliferative that is necessary for cardiac repair after ischemic injury.

microRNA can regulate CM cell cycle arrest, CM proliferation and cardiac repair after MI

MicroRNA (miRNA) are small, 22 nucleotides long single-stranded noncoding RNA molecules that regulate gene expression at the post-transcriptional stage [158]. MiRNA can silence protein expression through complete or partial interaction with complementary sequence of target mRNA. Such binding leads to either degradation or translational repression of the mRNA. A given miRNA can influence hundreds of genes, whereas a group of miRNAs can target the same mRNA [159]. The interaction between various combinations of miRNA with their target mRNA allows a fine-tuned regulation of their biological effect. Reportedly, the dysregulation of miRNA biogenesis and function can lead to cancer, neuropathology and heart disease [160,161]. miRNAs of the cardiovascular system were shown to control a wide range of biological functions, including CM proliferation, apoptosis and cardiac repair [162,163].

Overexpression and deletion studies of cardiac miRNA provided important information regarding the role that miRNA play during embryonic and postnatal development [164]. Dicer is a key enzyme necessary to produce mature miRNAs. CM-specific Dicer KO embryos generated with the Nkx2.5 promoter die by day E12.5 caused by an

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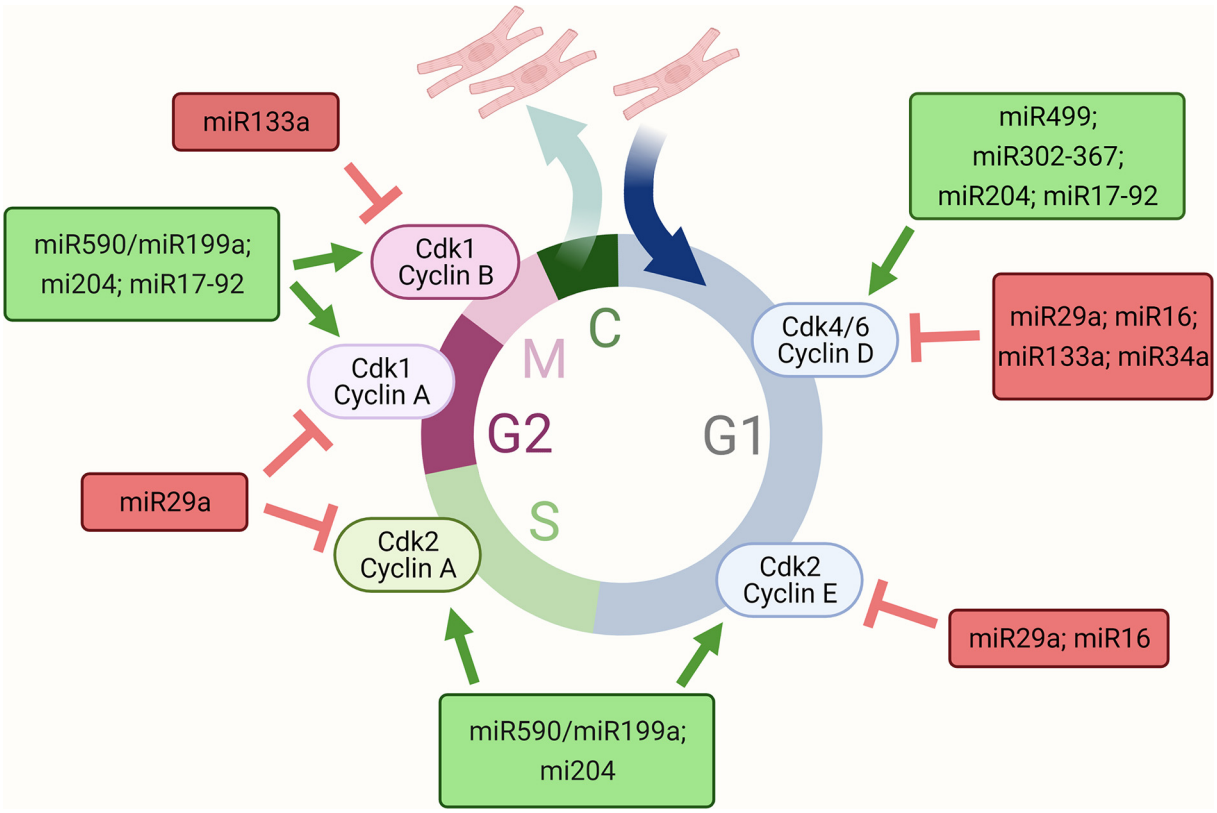

Figure 4. MicroRNAs involved in regulating CM cell cycle protein expression

CM expression of MicroRNAs (miRNA) are different during fetal and postnatal heart development. Multiple miRs regulate the cell cycle at various phases. MiRs that positively regulate CM cell cycle include miR499, miRNA302-367, miR204, miR17-29 and miR590/miR199a. Mi499 and 302-367 promote CM proliferation by up-regulating cyclin D expression which is prominent during G1-phase of the cell cycle. MiR302-367 does this by repressing the Hippo pathway. MiR204 up-regulates many cell cycle regulators including cycling A, cycling B, cyclin D2, cyclin E, Cdk1 and PCNA. Members of the miRNA17-92 cluster enhance the progression of the cell cycle by up-regulating cyclin B1, cyclin D1 and Cdk1. Cyclin B1 and Cdk1 are important factors that initiation of mitosis. MiR590/miR199a can both increase the expression of cyclins A, E and B in proliferating CM. MiRs that negatively regulate the cell cycle include miR29a, miR16, miR133a and miR34a. miR29a causes cell cycle arrest by targeting cyclin D, cyclin A and Cdk2, thus decreasing CM proliferation. MiR-16 down-regulates cyclin D and cyclin E expression and inactivates of cyclin/Rb pathway *in vitro*. MiR133a supresses CM proliferation by repressing cyclin D and cyclin B1 expression. The green color box/arrow represent miRs that promote CM proliferation. The red box/block arrow represent miRs that inhibit the CM cell cycle regulators. Created with BioRender.com

underdeveloped ventricular myocardium and pericardial edema. In contrast, Dicer KO mice developed dilated cardiomyopathy, heart failure and succumbed to early neonatal death [165]. Combined, these studies strongly suggest an important role of miRNA in the regulation of cardiac development and function.

In a different study, CM-specific overexpression of Dicer in 3-week-old mice resulted in rapid death within one week [166]. Heart of these exhibited mild ventricular remodeling, atrial enlargement, and inflammatory responses. In contrast, CM-specific removal of Dicer in adult hearts caused spontaneous cardiac hypertrophy, ventricular remodeling with dilation and heart failure [166]. These findings support the view that biogenesis of miRNA is crucial to maintain a proper architecture and function of the adult heart.

Reportedly, miRNA can also modulate the expression of many cell cycle regulators involved in the control of CM proliferation. A high-throughput screening identified as many as 204 miRNAs that can enhance neonatal CM cell cycle entry together with 331 miRNA that inhibited this process [166]. Based on gain- and loss-of-function experiments, miRNAs 16, 29, 99, 133 and 195 were identified as potent regulators of cell cycle factors (Figure 4) [167]. Likewise, miRNA cluster 302-367 and 17-92 were shown to participate in the regulation of CM proliferation in embryonic,

postnatal and adult hearts. miRNA 302-367 promotes CM proliferation by repressing the Hippo activity through inhibition of Mst1, Lats2 and Mob1b thereby rising cyclin D expression [168]. In contrast, expression of miRNA-19a/19b, members of the miRNA17-92 cluster, led to induction of CM proliferation through up-regulation of cyclin B1, D1 and Cdk1 [169]. Increased expression of these cell cycle protagonists was associated with down-regulation of Pten, a major inhibitor of the Pi3K-Akt pathway.

Other miRNAs including miRNA-499, miRNA-590 and miRNA-199a also have the ability to mediate cell cycle re-entry in neonatal and adult CM *in vivo* [170]. Cardiac injections of synthetic miRNA mimics to miRNA-590/199a into the hearts of neonatal mice led to increases in the number of CM in S-phase. Moreover, AAV-mediated transduction of miRNA-590 or miRNA-199a in both neonatal and adult mice also elevated CM proliferation contributing to improved heart function post-MI [170].

In addition, CM-specific overexpression of miRNA-204 in isolated neonatal rat CM *in vitro* and in the heart of transgenic rat *in vivo* induces CM proliferation [171]. Increases in miRNA-204 levels were associated with higher levels of G1-phase factors D2/E, G2/M-phase cyclins A/B and Cdk1. Moreover, the authors demonstrated that miRNA-204 directly inhibits the transcription repressor Jumanji-AT rich interactive domain (Jarid2) to induce the expression of these positive cell cycle factors regulators [171].

Reportedly, several miRNAs exert an inhibitory impact on CM proliferation by downregulating cell cycle activators. The miRNA-15 family members are up-regulated in the early neonatal period where they cause CM mitotic arrest [172]. Additionally, overexpression of miRNA-195, another miRNA-15 family member, inhibits checkpoint kinase (Chek1) expression thereby causing a cell G2/M cycle arrest. Antagomir-dependent inhibition of miRNA-15 in postnatal mice is linked with an increased number of mitotic CMs and Chek1 expression [172]. miRNA-16, another member of miRNA-15, is specifically down-regulated in hypertrophic mouse and rat hearts [173]. Remarkably, overexpression of miRNA-16 inhibited the development of cardiac hypertrophy through activation of the Rb pathway and inhibition of cyclin D/E. Similarly, miRNA-29a inhibits CM division by lowering expression levels of Cdk2 and cyclin D/A [174]. Antagomir-mediated inhibition of miRNA-29a is able to restore proliferation of CM proliferation [175].

Mice lacking miRNA-133a exhibit excessive CM proliferation, whereas CM-specific overexpression of miRNA-133a in transgenic mice inhibits proliferation [176]. Reportedly, this miRNA can suppress CM proliferation by repressing cyclin D/B1 expression. Cyclin D1 is also negatively regulated by miRNA-34a [177]. miRNA-34a levels are low in embryonic and postnatal hearts but are increased at 7d post-birth and remain high throughout adulthood. Delivery of antagomirs to miRNA-34a diminished miRNA-34a levels and contributed to improved cardiac remodeling and function post-MI. The anti-proliferative action of miR-34a was mediated directly though inhibition of BCL2 apoptosis regulator (Bcl2), cyclin D1, and sirtuin (Sirt1) [177].

In summary, all these findings are in favor with the concept, that miRNA are important cell cycle regulators in CM. Modulation of miRNA levels could be harnessed as a novel strategy to treat heart failure in the clinic.

p53/Mdm2 synergistically regulate a network of microRNAs to maintain cell cycle arrest in CM

One of the key activities through which p53 induces cell cycle arrest in the G1-phase and apoptosis is through the transcriptional activation of cell cycle inhibitory p21, and BCL2 apoptosis regulator (Bbc3), BCL2-binding component 3 (Puma) and phorbol-12-myristate-13-acetate-induced protein (Noxa) [178,179]. Mice with germline deletion of p53 are prone to tumorigenesis around 6-8 weeks after birth [180]. We and others have previously shown that deletion of p53 is an important factor in the development of dilated cardiomyopathy post-MI [181]. Moreover, our group has shown that CM-specific genetic ablation of p53 in adult mice confers cardio-protection through resistance to acute biomechanical stress [182].

It has been well established, that p53 activity is mainly controlled post-translationally through transformed mouse 3T3 cell double minute (Mdm2) [183]. Mdm2 is an E3 ubiquitin ligase that ubiquitinates p53 inducing its proteasomal degradation [184]. Germline Mdm2 KO mice exhibit a very early embryonic lethality, due to high levels of p53, that causes an excessive apoptosis with diminished cell proliferation [185]. The cardiac-specific ablation of Mdm2 in adult mice leads to cardiac hypertrophy with extensive apoptosis and ventricular remodelling, heart failure and early death [186]. Intriguingly, cardiac-specific conditional DKO of p53 and Mdm2 in adult mice induced a 50% increase in CM number. Mechanistically, CM proliferation was accompanied by down-regulation of p21/p27 and induction of Cdk2. Moreover, miRNA expression profiles revealed that 11 miRNA targeting cell cycle activators were specifically down-regulated in DKO hearts [186]. Combined, these results indicate that p53/Mdm2 regulate a miRNA network that serves as a barrier against CM proliferation to maintain cardiac homeostasis.

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The MAP-kinase p38α is a key inhibitor of CM proliferation

Surprisingly little is known about the mechanistic role of mitogen-activated protein kinase 14 (p38α) in cardiac regeneration. p38α activation could inhibit proliferation of adult zebrafish CM [187]. During heart regeneration in adult zebrafish, the induction of p38α activity blocks CM proliferation, suggesting that p38α activity must be switched off to trigger CM proliferation [188,189]. Intriguingly, this mechanism was also well documented in neonatal and adult rat CM, both *in vitro* and *in vivo* [188,190].

Mammalian hearts have a very low or non-existent regenerative capacity after cardiac injury [126]. Nevertheless, in principle, signals that acutely trigger CM survival or modulate myoblast activity could be manipulated to promote cardiac regeneration and avoid heart failure. There is evidence implicating fibroblast growth factor (Fgf1) up-regulated genes in cardiac regeneration and cell cycle control. This inhibition of p38α and stimulation of Fgf1 act together to induce the expression of specific genes involved in proliferation and regeneration, such as cytokinesis regulator Ect2, CCAAT enhancer binding protein epsilon (Crp1), marker of proliferation Ki-67 (Ki67), Cdk1, cyclin A and p27 [126]. Moreover, p38α inhibition and Fgf1 induction leads to inactivation of Rb, a major cell-cycle inhibitor in CM. The promotion of CM proliferation by combined treatment with Fgf1 and a p38 inhibitor in the adult rat heart post-MI demonstrates that this combination treatment could provide a novel therapeutic approach to preserve cardiac function after injury [127] .

Meis1 controls perinatal CM proliferation and cell-cycle exit

The Meis homeobox (Meis1) gene product is one of the few transcription factors that impact the expression of cell-cycle regulatory factors in CM [191]. Meis1 is a homeodomain protein which is highly expressed in postnatal CM where it regulates cell cycle exit and regenerative capacity. CM-specific ablation of Meis1 extended the postnatal CM proliferative window from 7 to 14 days [192]. Moreover, genetic deletion of Meis1 in adult CM induced proliferation in the absence of hypertrophy or impaired cardiac function. In contrast, overexpression of Meis1 in neonatal CM reduced proliferation. Mechanistically, Meis1 deletion down-regulated the Cdkis p15, p16 and p19, together with the Kip-family members p21 and p57. Since the gene promoters of these cell cycle inhibitors contain consensus-binding sites for Meis1, it was suggesting that Meis1 directly participates in their transcriptional regulation [192]. Collectively, Meis1 inhibits postnatal cardiac regeneration by promoting CM cell cycle exit in the early neonatal period through activation of Cdki [193].

Fstl1 enables CM proliferation and cardiac repair

The cardiac epicardium is an external epithelial layer that enables embryonic heart growth through growth factors directed activation of progenitor cells [194]. The elucidation of epicardial factors that can trigger proliferation of adult CM and repair the adult heart is of potential clinical importance. Reportedly, follistatin like (Fstl1) is expressed in epithelial cells in normal hearts, where it exerts an important function in regulation of proliferation [195]. Epithelial Fstl1 protein levels decline whereas they increase in CM post-MI. However, Fstl1 expression in CM fails to promote CM proliferation post-MI and normal hearts exposed to ectopic expression of Fstl1 [196]. These results provide experimental evidence that a maladaptive process abolishes the proliferative capacity of CM in response to ischemic injury. In contrast, cardiac delivery of recombinant Fstl1 through an epicardial patch model stimulates CM proliferation in normal hearts. Importantly, application of a recombinant Fstl1-patch led to significantly improved heart function in mouse and swine post-MI. Therefore, the loss of epicardial Fstl1 contributes to HF post-MI [196]. Restoration of epicardial Fstl1 function by delivery of a recombinant protein version could prevent deterioration of heart function post-MI. This effect of recombinant Fstl1 is potentially amenable to clinical translation.

ECM remodeling with Agrin induces CM proliferation

Heart failure post-MI involves extensive remodeling of the ECM and scarring. Cytoplasmic membrane-bound dystrophin connects ECM components such as agrin and laminin to the cytoskeleton [197]. Agrin is a large ECM heparan sulfate proteoglycan and interacts with dystrophin [198]. Interestingly, agrin-binding to dystrophin promotes disassembly of the ECM. In the neonatal heart, elevated agrin levels allow dystrophin-binding to the ECM, that presumably contributes to CM cell cycle exit shortly after birth [199]. Agrin KO mice die around birth due to disturbances in neuromuscular function. Administration of agrin in adult mice induced adult CM proliferation post-MI. While this impact of agrin on cell cycle re-entry is astonishing, the authors concluded that the low numbers of actively dividing CM indicate that additional mechanisms must contribute to cardiac repair observed in their agrin model

system [199]. In summary, this study delivered sustained experimental *in vivo* evidence that mammalian heart repair post-MI involves destabilization of the ECM.

Conclusions

CM proliferation is a tightly regulated process involving a complex regulation of signal transduction pathways. Uncovering the molecular mechanisms that promotes proliferation in the embryonic heart has been key to the identification of potential candidates that maybe helpful in the development of novel clinical treatment options post-MI. As delineated in our review, many experimental studies document the beneficial impact of targeting the cell cycle to induce CM proliferation post-MI. This review also demonstrates that many signaling pathways such as Wnt-Ctnnb1, Hippo, Pi3K-Akt, Rb-E2f or p53-Mdm2 are involved in the regulation of cell cycle re-entry and progression in adult mammalian CM.

Several miRNAs can specifically trigger or inhibit CM proliferation in neonatal and adult hearts suggesting that synthetic miRNA mimics could besuited as a clinical strategy for cardiac repair after ischemic injury. Clearly, more research is needed to fill the gaps cell cycle entry in big mammals and in the adult human heart.

Many results in the field of CM proliferation were obtained employing mouse models of myocardial infarction [200]. In this model systems, it has remained controversial, whether genetic manipulation of gene with or without an additional stimulus such as growth factors, promotes CM cell cycle entry and induces CM division. It is undisputed that ectopic overexpression of pro-proliferative factors in immature neonatal rat and mouse CM can efficiently promote CM proliferation. However, the data on induction of adult CM proliferation often appear rather weak. Moreover, the observed improved heart function post-MI might not be the sole effect of CM proliferation since this can also be explained by other effects, such as enhanced angiogenesis during ventricular remodeling post-MI.

Despite immense research in cardiovascular regenerative medicine, the degree of repair through CM proliferation as observed in the newt has not been achieved in other small animal models. For many investigations, it has also remained unclear whether a stimulus that evokes CM proliferation is indeed solely responsible for cardiac repair. Lastly, there remain many technological challenges that relate to the development of standardized assays, such as immunofluorescence microscopy procedures that unequivocally identify dividing CM *in vivo* in order to minimize discrepancies and improve reproducibility and predictability. There is also no doubt that it will be a challenging task to sort out and exploit the beneficial activities of CM proliferation while limiting their side effects for clinical therapies.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Donya Mahiny-Shahmohammady: Conceptualization, Writing—original draft, Writing—review & editing. **Ludger Hauck:** Conceptualization, Supervision, Writing—original draft, Writing—review & editing. **Filio Billia:** Conceptualization, Supervision, Writing—original draft, Writing—review & editing.

Abbreviations

Cdk, cyclin-dependent protein kinase; Chek1, checkpoint kinase; CM, cardiomyocytes; DDR, DNA-damage response; ECM, extracellular matrix; GPCR, G-protein-coupled receptors; Fgf1, fibroblast growth factor; Igf-1, insulin-like growth factor; Nrg1, Neuregulin-1; PDK4, pyruvate dehydrogenase kinase 4.

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