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Harnessing the Induction of Cardiomyocyte Proliferation for Cardiac Regenerative Medicine

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Keywords

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Introduction

Cardiovascular disease is the leading cause of death in the United States and a growing health problem world-wide[1]. Following an adverse event such as myocardial infarction, there is a loss of nearly 1 billion cardiomyocytes that results in significant compromise to cardiac function. In humans, unlike some lower organisms such as the newt and zebrafish, these lost cardiomyocytes are not fully replenished through division of existing adult myocytes[2, 3]. Adult human cardiomyocytes are among the handful of cell types in the human body with extremely limited mitotic potential. Hence, the postnatal heart largely increases its size through hypertrophy and not hyperplasia[4]. Interestingly, studies in mouse models have demonstrated that the mammalian heart exhibits a robust regenerative response during embryonic development but this ability is quickly lost after birth and declines further with adult maturation[5, 6]. Some investigators have reported the possibility of adult human myocyte replenishment through the division of rare populations of adult cardiac stem cells[7] or specialized populations of mitotically active myocytes[8]. While the precise contribution to new adult cardiomyocytes by these specialized populations of cells remains to be determined, their effect on cardiomyocyte replacement after injury is expected to be low and insufficient to compensate for the one billion cardiomyocytes lost during myocardial infarction. Consequently, the replenishment of lost cardiomyocytes in adult heart, remains a major unmet need in cardiovascular regenerative medicine.

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Conflict of Interest

None of the authors declare a conflict related to this paper.

Modern post-infarction treatment options target the neurohormonal physiology of residual cardiomyocytes without attempting to replenish the lost myocytes. For example, standard treatment regimens for post-MI heart failure due to cardiomyocyte loss include beta blocker, ACE inhibitors, and diuretics to 1) reduce oxygen demand, sympathetic overstimulation, and arrhythmias by managing heart rate and blood pressure, 2) reduce activation of the renin-angiotensin-aldosterone pathway, and 3) improve symptoms by managing the body fluid volume overall. In the most extreme cases of post-infarction heart failure where medical therapy is inadequate, replacement of the whole organ function by left ventricular assist device (LVAD) or heart transplantation may be necessary[9]. However, heart transplantation is associated with a host of complex issues, the most pressing of these being the limited availability of replacement organs and significant financial costs associated with such procedures[10]. As such, novel approaches for restoring cardiac function following myocardial infarction are in dire need.

Current Applications of Human Pluripotent Stem Cells for Studying Cardiomyocyte Proliferation and Cell Therapy

Recently, advances in stem cell biology and regenerative medicine have allowed, for the first time, mass production of human cardiomyocytes from pluripotent stem cells. Early cardiomyocyte differentiation protocols were limited in terms of their ability to create large numbers of cardiomyocytes as they employed cumbersome embryoid body-based methods and expensive growth factor treatment regimens[11, 12]. Current differentiation protocols can generate millions of cardiomyocytes from both human embryonic stem cells and patient specific human induced pluripotent stem cells using chemically-defined and small molecule-based approaches[13, 14]. Future refinements in methods for pluripotent stem cell culture and differentiation should easily lead to the production of billions of cardiomyocytes within two to three weeks from the onset of differentiation[15]. Although these stem cell-derived cardiomyocytes exhibit fetal-like phenotypes in terms of their structural and electrophysiological properties, they are currently the only reliable source of human cardiomyocytes that can be used to study human cardiovascular disease mechanisms[16]. For example, human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have been shown to recapitulate the molecular mechanisms driving cardiovascular diseases such as long QT syndrome, viral myocarditis, and various other types of cardiomyopathies[17–19]. Additionally, because cardiotoxicity is the leading cause of drug withdrawal from the market, there is much interest within the pharmaceutical industry for using hiPSC-CMs for high throughput cardiotoxicity screening[20]. Finally, early stage clinical trials focusing on directly using stem cell-derived cardiomyocytes for regenerative therapy following myocardial infarction are underway. A recent study demonstrated in a non-human primate model that stem cell-derived cardiomyocytes are able to integrate into the host heart for a number of months[21]. Additionally, bioengineering-based approaches, utilizing stem cell-derived cardiomyocytes in combination with various biocompatible scaffolding technologies such as patches, are being tested as a way to introduce cardiomyocytes into damaged areas of the myocardium following infarction[22, 23]. Such engineered heart muscle could also be used for in vitro drug screening to test the efficacy or toxicity of compounds, with the assumption that 3D tissue slices made from PSC-CMs may be more likely to predict the

behavior of three-dimensional tissue structure of the native heart. Given these possibilities, there is much hope that stem cell-derived cardiomyocytes can be used for 1) cardiovascular disease modeling, 2) drug toxicity screening, and 3) cell therapy (Figure 1).

Screening for Novel Regulators of Cardiomyocyte Cell Cycle Activity

Beyond the aforementioned applications, PSC-CM could also be used to screen for novel regulators of cardiomyocyte proliferation. A number of studies have previously shown that cardiomyocytes can exhibit cell cycle activity in response to treatments with an array of compounds in vitro and in vivo (Table 1).

Treatments with extracellular ligands, secreted hormones, growth factors have been shown to activate critical signaling pathways that up-regulate cell cycle activity in cardiomyocytes. Insulin-like growth factor (IGF) enhances proliferation of terminally-differentiated human embryonic stem cell-derived cardiomyocytes through activation of pro-proliferative PI3K/Akt signaling, as well as promotes expansion of early mesoderm during cardiomyocyte differentiation in mouse embryonic stem cells[24, 25]. Periostin, a ligand for the alpha-V/beta-3 and alpha-V/beta-5 integrins, was reported by Kuhn et al to induce cell cycle reentry of differentiated myocytes in vitro and promote mononucleated myocytes to enter mitosis via activation of PI3K signaling. Remarkably, this study showed that periostin treatment results in enhanced myocardial function post-infarction in rat hearts[26]. Bersell et al demonstrated that Neuregulin 1 stimulates cell cycle reentry, karyokinesis, and cytokinesis of rat ventricular cardiomyocytes by activating tyrosine kinase signaling downstream of the ErbB4 receptor[27]. However, the validity of these results is actively debated[28]. Nevertheless, neuregulin treatment was reported to improved cardiac function post-infarction[27]. Alternatively, thyroid hormone treatment has been reported to induce cardiomyocyte proliferation[29]. A thyroid hormone surge during neonatal development was reported to be able to induce a proliferative burst in predominantly binuclear cardiomyocytes via activation of the IGF signaling pathway. Low levels of the cardiac natriuretic peptides ANP and BMP were also shown to enhance CM proliferation via activation of Npr3 and cAMP pathways in vitro in neonatal rat ventricular myocytes and also in the zebrafish heart in vivo[30]. In rat myocytes, stromal cell-derived factor 1 alpha enhanced the proliferation ability of cardiomyocytes via the PI3K/Akt and ERK signaling pathway[31]. More recently in human iPSC-CMs and primate ESC-CMs, granulocyte colony stimulating factor (GCSF) was shown to promote proliferation of developing CMs by activating the JAK/STAT signaling pathway[32]. Additionally, intrauterine GCSF injection promoted cardiomyocyte hyperplasia in developing mouse embryos[32]. Cytokine interleukin 13 stimulated cell cycle reentry of neonatal rat ventricular cardiomyocytes and preferentially induced either proliferation or survival of CMs compared with other cell types[33]. TGF-beta superfamily growth factor BMP10 induced dose-dependent CM proliferation and cell cycle reentry of differentiated rat ventricular cardiomyocytes in vitro, and stimulated CM cell-cycle reentry and mitosis post-infarction in vivo by enhancing expression of the cardiac transcription factor Tbx20[34]. Similarly, paracrine factor C3orf58, a hypoxia and Akt induced stem cell factor, was shown to enhance cardiac repair in vivo and a 60% increase in DNA synthesis in neonatal rat ventricular myocytes[35]. Finally, FGF ligands were able to enhance regional CM proliferation in the fetal murine heart via the FOXO3/p27 signaling pathway, and their overexpression in

adult mice promotes myocyte cell cycle reentry[36, 37]. These studies demonstrate that extracellular ligands, secreted hormones, and growth factor treatments are able to exert a proliferation-enhancing effect in mammalian cardiomyocytes both in vitro and in vivo.

For translational purposes, small molecules may be preferable as agents for stimulating cardiomyocyte proliferation as they are relatively cheap in comparison to growth factors and, as synthetic molecules, can be produced under xeno-free conditions in a GMP compliant fashion. For example, Engel et al demonstrated that the small molecule p38 inhibitor SB203580 induced DNA synthesis, karyokinesis, cytokinesis of adult rat ventricular CMs through dedifferentiation of sarcomeric structures[38]. Likewise, BIO (6-bromoindirubin-30-oxime), a GSK3beta inhibitor, promoted cell cycle reentry and progression and increased mitosis through activation of the canonical Wnt pathway in multiple cardiomyocyte types[39, 40]. Uosaki et al also demonstrated that small molecules SU1498 and KN93 increased PSC-CM proliferation through activation of the Raf-MEK-ERK signal cascade and inhibition of CAMKII, respectively[40]. These studies demonstrate that small molecules are effective and stable regulators of myocyte cell cycle activity and some may even improve the cardiomyocyte yield in chemically-defined cardiomyocyte differentiation protocols[14].

MicroRNAs have recently garnered much interest for their abilities to control gene expression via RNA silencing. Multiple miRNA families have been studied in the context of cardiac development, and some are noted for their abilities to promote myocyte replication in vitro and in vivo. Knockout of miR-133a was able to enhance CM proliferation through elevation of cyclin D2 in the mouse heart[41]. A functional screen identified that miR-199a-3p and miR-590-3p were able to induce cell cycle reentry and cytokinesis of mouse adult and neonatal cardiomyocytes in vitro and in vivo following MI[42]. Knockdown of the miR-15 family increased mitotic activation of cardiomyocytes via de-repression of Chek1[43]. Overexpression of miR-17-92 was sufficient to promote CM proliferation in vitro and in vivo in rat and mouse CMs by repressing the tumor suppressor PTEN[44]. MiR-499 was able to enhance neonatal rat ventricular CM proliferation in vitro through an effect on Sox6 and cyclin D1[45]. The reactivation of cell cycle activity in postnatal mouse CMs and reduction of scar formation was achieved by increasing the expression of miR302-367 in vivo. Interestingly, this activity of miR302-37 required the presence of multiple members of the Hippo signaling pathway[46]. Treatment with anti-miR-34 was able to enhance post-MI remodeling in the adult murine heart by inhibiting the function of miR-34 during apoptosis through its target genes Bcl2, Cyclin D1, and Sirt1[47]. Finally, mir-99/100 and Let-7a/c and their downstream targets smarca5 and FNTB were identified as regulators of heart regeneration in zebrafish and overexpression of these target proteins enhanced the proliferative response in murine models[48]. These studies illustrate that miRNAs, a relatively new topic in cardiac biology, play a significant role in regulating cardiomyocyte proliferation.

In addition to exogenous introduction of pro-proliferative proteins and other factors, a number of studies have utilized genetic manipulation of critical cell cycle regulating pathways to hyperactivate cardiomyocyte proliferation. While genetic overexpression of pathways that enhance cardiomyocyte proliferation may not be directly translatable to human therapy, it can provide important mechanistic insights for studying the signaling

network regulating cardiomyocyte proliferation. Overexpression of a number of cyclin proteins and cyclin dependent kinases has proven effective in enhancing cardiomyocyte proliferation in mouse models as well as in in vitro systems. Bicknell et al demonstrated that overexpression of CCNB1 induced multinucleation, cell cycle reentry, as well as DNA synthesis in adult rat cardiomyocytes[49]. Likewise, overexpression of Cyclins A2, D1, D2, and others induced postnatal mitosis of adult myocytes and functional benefit after myocardial infarction in mouse models[50–53]. Deletion of the FOXO1 transcription factor was able to downregulate cyclin kinase inhibitor expression, thereby increased myocyte proliferation[54]. Similarly, knockout of the Jumonji protein was able to increase myocyte mitosis in primary mouse cardiomyocytes by upregulating cyclins D1 and D2[55]. Taken together, these studies illustrate that cardiomyocytes are susceptible to cell cycle reentry following stimulation with a number of exogenous factors.

Conclusions

Effective induction of proliferation in adult human cardiomyocytes represents a significant goal for the cardiovascular regenerative medicine community. A number of lower vertebrates, adult zebrafish[3], and neonatal mouse hearts[5] have all been shown to exhibit significant cardiac regenerative potential following experimental induction of myocardial damage, raising the possibility that with the right mechanistic insight a similar process may be recapitulated in humans. Other studies have demonstrated the effects of cell cycle activating compounds, identified through in vitro myocyte screening, to enhance cardiomyocyte division in animal studies in vivo. Alternatively, direct reprogramming of existing cardiac fibroblast population in the mammalian heart both in vitro[56] and in vivo towards the cardiomyocyte lineage has been reported to work in murine models, although at an extremely low efficiency. Because of recent advances in cardiomyocyte differentiation protocols from pluripotent stem cells, these cells can now be mass-produced for the screening of novel regulators of cell cycle activity. We believe that human stem cell-derived cardiomyocytes, because of their similar gene expression pattern and cell cycle regulation to primary human cardiomyocytes, represent the best current surrogate cell type for understanding human cardiomyocyte biology and cell cycle induction. Still, much work remains to be done with regards to PSC-CM cell production and maturation. Advancements must still be made in scale of production as well as reproducibility of cardiomyocyte differentiation from pluripotent stem cells, as cardiomyocyte differentiation yield remains variable from stem cell line to stem cell line. Additionally, developing directed differentiation protocols for production of atrial, ventricular, and nodal-specific cardiomyocyte subtypes would be valuable, as different cardiomyocyte populations may respond differently to treatment with proliferation-inducing compounds. Finally, because current cardiomyocyte differentiation protocols produce PSC-CMs that are fetal-like in their gene expression patterns and structure, induced maturation of these cells will be necessary in order to accurately model cardiovascular disease phenotypes and cardiotoxic drug responses. Likely, these issues will be addressed, as cardiomyocyte differentiation protocols and developments in iPSC reprogramming have been advancing at breakneck speed. In spite of their present limitations, we ultimately believe that stem cell derived cardiomyocytes

represent an excellent platform for screening for novel regulators of cardiomyocyte proliferation, which could eventually be utilized for translational therapies.

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Opinion Statement

Adult human cardiomyocytes are terminally-differentiated and have limited capacity for cell division. Hence, they are not naturally replaced following ischemic injury to the heart. As such, cardiac function is often permanently compromised after an event such as myocardial infarction. In recent years, investigators have focused intensively on ways to reactivate cardiomyocyte mitotic activity in both in vitro cell culture systems and in vivo animal models. In parallel, advances in stem cell biology have allowed for the mass production of patient-specific human cardiomyocytes from human induced pluripotent stem cells. These cells can be produced via chemically-defined differentiation of human pluripotent stem cells in a matter of weeks and could theoretically be utilized directly for therapeutic purposes to replace damaged myocardium. However, stem cell-derived cardiomyocytes, like their adult counterparts, are post-mitotic and incapable of large-scale expansion after reaching a certain stage of in vitro differentiation. Due to this shared characteristic, these stem cell-derived cardiomyocytes may provide a platform for studying genes, pathways, and small molecules that induce cell cycle reentry and proliferation of human cardiomyocytes. Ultimately, the discovery of novel mechanisms or pathways to induce human cardiomyocyte proliferation should improve our ability to regenerate adult cardiomyocytes and help restore cardiac function following injury.

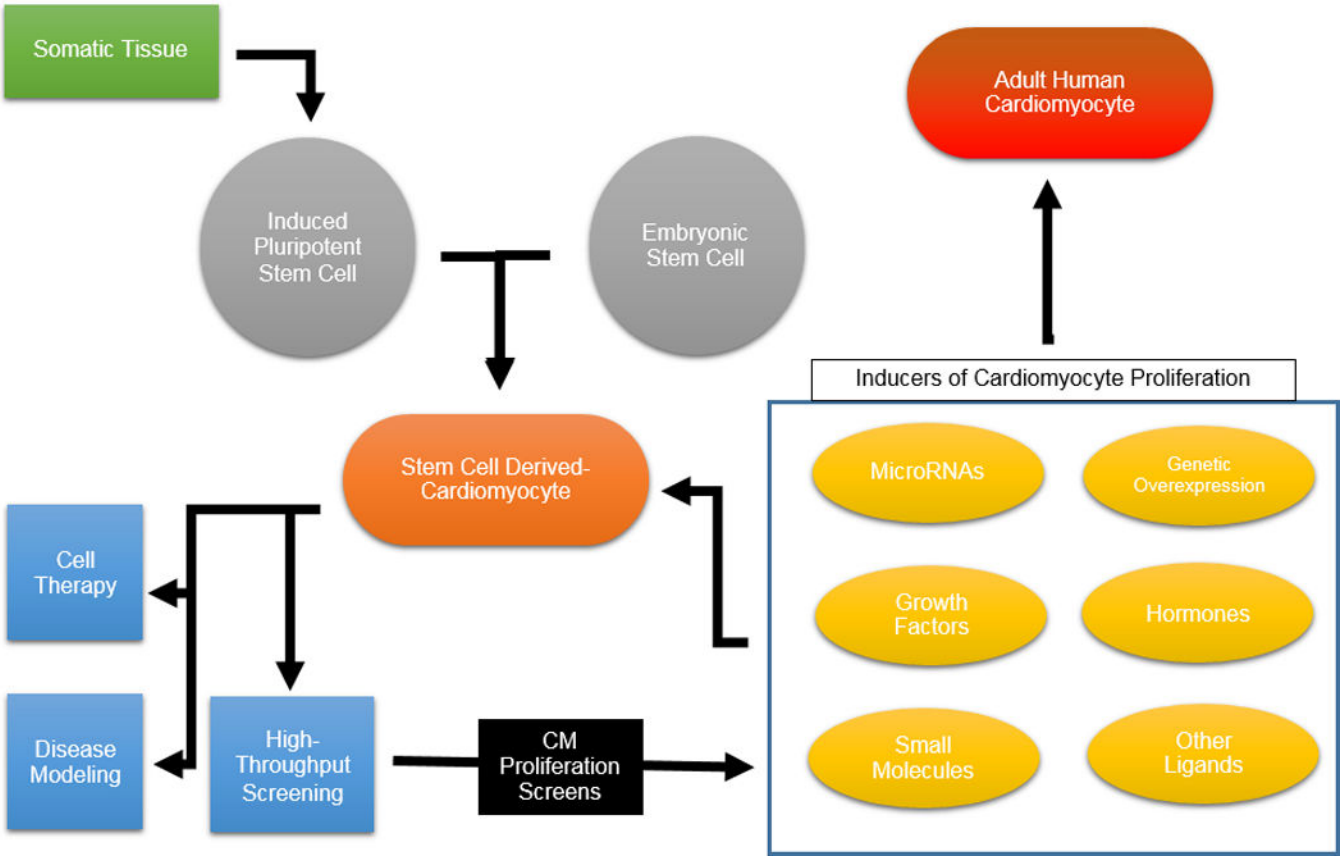

Figure 1. Applications of stem cell-derived cardiomyocytes

Human pluripotent stem cell-derived cardiomyocytes can be produced from induced pluripotent stem cells derived from somatic tissue or from embryonic stem cells. Following differentiation, PSC-CMs can be used for cardiovascular disease modeling, potentially for cell therapy, or for high-throughput screening purposes. As an extension of high-throughput screening applications, PSC-CMs can be utilized to identify novel regulators of cardiomyocyte proliferation. These pro-proliferative compounds could then be tested on adult human cardiomyocytes.

Table 1

Studies of cardiomyocyte cell cycle reentry in in vitro platforms and animal models

Name	In Vivo/ In Vitro	Summary	Citation
ANP/BNP	In vitro (neonatal rat ventricular CMs) In vivo (zebra fish)	Low levels of the natriuretic peptides enhances CM proliferation via activation of Npr3 and cAMP pathways.	Becker et al., 2013
BMP10 (Bone morphogenetic protein-10)	In vitro (rat ventricular CMs) In vivo (rat)	BMP10 induces dose-dependent CM proliferation and cell cycle reentry of differentiated CMs in vitro, and stimulates CM cell-cycle reentry and mitosis post MI in vivo, by enhancing Tbx20 expression.	Sun et al., 2014
Cyclin A2	In vivo (mouse)	Cyclin A2 induces postnatal mitosis, stimulates CM proliferation, and provides benefit after myocardial infarction.	Chaudhry et al., 2004; Woo et al., 2006
Cyclin B1	In vitro	Overexpression of Cyclin B1 induces cell cycle reentry, DNA synthesis, and multinucleation.	Bicknell et al., 2004
Cyclin D1	In vitro (neonatal rat CMs) In vivo	Nuclear import of cyclin D1 and its partner CDK4 promotes cell-cycle reentry by activating the Rb regulatory pathway, which is required for cell cycle progression in both fetal CMs and adult heart.	Tamamori-Adachi et al., 2003
Cyclin D2	In vivo (mouse)	Cyclin D2 promotes CM DNA synthesis in uninjured hearts and promotes regression growth post MI.	Pasumarthi et al., 2004
C3orf58 (Hypoxia and Akt induced stem cell factor, HASF)	In vitro (neonatal rat ventricular CMs) In vivo (mouse)	HASF increases DNA synthesis and promotes mitosis and cell division in vitro and enhances DNA synthesis and karyokinesis of neonatal and adult CMs in vivo through the PI3K/Akt-dependent CDK7 pathway.	Beigi et al., 2015
FOXO 1	In vitro (mouse embryonic CMs)	Absence of FOXO 1 downregulates cyclin kinase inhibitor expression and increases CM proliferation.	Evans-Anderson et al., 2008
JMJ (Jumonji)	In vitro (primary mouse CMs)	JMC knock-out increases mitosis via derepression of cell cycle progression by the interaction between JMC and Rb, with up-regulation of cyclin D1, cyclin D2, and Cdc2.	Jung et al., 2005
IL13 (Interleukin 13)	In vitro (neonatal rat CMs)	IL13 stimulates cell cycle reentry of CMs and preferentially induces either proliferation or survival of CMs compared with other cell types.	O'Meara 2015
FGF-1 (Fibroblast growth factor-1)	In vitro (rat neonatal ventricular CMs) In vivo (mouse)	FGF-1 mediates CM cell cycle reentry through TWEAK/Fn14 and FGF1/FGFR-1 signaling cascades crosstalk.	Novoyatleva et al., 2014
FGF-10	In vivo (mouse)	FGF10 regulates regional CM proliferation in the fetal heart through a FOXO3/p27 (kip1) pathway, and its overexpression in adult mice promotes CM but not cardiac fibroblast cell-cycle re-entry.	Rochais et al., 2014
G-CSF (Granulocyte colony stimulating factor)	In vitro (primate ESC-CM, human iPSC-CM) In vivo (mouse)	G-CSF promotes proliferation of developing CMs by activating the JAK/STAT signaling pathway.	Shimoji et al., 2010

Name	In Vivo/ In Vitro	Summary	Citation
IGF1/2 (Insulin-like growth factor 1/2)	In vitro (hESC-CM)	IGF1 or IGF1 stimulates proliferation of terminally differentiated hESC-CMs in a dose-dependent manner by triggering PI3K/Akt signaling.	McDevitt et al., 2005
IGF1/2	In vitro (murine ESCs)	IGF treatment during early differentiation of ESCs increases mesodermal cell proliferation and, consequently, CPC formation through activation of downstream targets such as Akt and mTOR.	Engels et al., 2014
NRG1 (Neuregulin1)	In vitro (rat ventricular CMs) In vivo (mouse)	NRG1 stimulates cell cycle reentry, karyokinesis and cytokinesis of differentiated CMs by activating tyrosine kinase signaling downstream of the ErbB4 receptor, and leads to improved cardiac function post MI.	Bersell et al., 2009
SDF-1α (Stromal cell-derived factor-1α)	In vitro (rat CMs)	SDF-1α enhances the proliferation ability of CMs with physical injury through regulating PI3K/Akt and ERK signaling pathway. Proliferation rate increases most markedly at 80 μg/L.	Hou et al., 2015
BIO (6-bromoindirubin-30-oxime)	In vitro (neonatal and adult rat CMs; mESC-CMs, hiPSC-CMs)	BIO, a GSK3B inhibitor, promotes cell cycle reentry and progression and increases the number of mitoses of CMs through activation of the canonical Wnt pathway.	Uosaki et al., 2013
KN93	In vitro (mESC-CMs, hiPSC-CMs)	KN93 increases CM proliferation by inhibiting CAMKII.	Uosaki et al., 2013
SB203580	In vitro (adult rat ventricular CMs)	SB203580 inhibits p38 MAPK, by which promotes growth factor induced DNA synthesis, karyokinesis, cytokinesis of CMs through dedifferentiation of sarcomeric structures.	Engel et al., 2005
SU 1498	In vitro (mESC-CMs, hiPSC-CMs)	SU1498 increases CM proliferation through activation of Raf-MEK-ERK signal cascade.	Uosaki et al., 2013
miR-15 family	In vivo (mouse)	Knockdown of the miR-15 family increases number of mitotic cardiomyocytes and derepression of Chek1.	Porrello et al., 2011
miR-17–29	In vitro (neonatal rat cardiomyocytes) In vivo (mouse)	Overexpression of miR-17–92 is sufficient to induce CM proliferation in embryonic, postnatal, and adult hearts by repressing tumor suppressor Phosphatase and Tensin Homolog (PTEN).	Chen et al., 2013
miR-34a	In vivo (mouse)	Anti-miR-34a treatment improves post-MI remodeling in adult heart by interfering with miR-34a's regulation of cell activity and death via its target genes including Bcl2, Cyclin D1, and Sirt1.	Yang et al., 2015
miR-99/100 and Let-7a/c and their downstream protein targets SMARCA5 and FNTβ	In vivo (mouse)	Anti-miR delivery or overexpression of FNTβ and SMARCA5 results in adult CM dedifferentiation and the activation of a proliferative response.	Aguirre et al., 2014
miR-133a	In vivo (mouse)	Knockout of miR-133a promotes CM proliferation by elevating expression of SRF and cyclin D2.	Liu et al., 2008
miR-199a-3p and miR-590-3p	In vitro (mouse and rat CMs) In vivo (mouse)	miR induces cell cycle reentry and cytokinesis of neonatal and adult cardiomyocytes in vitro and promotes CM proliferation in both neonatal and adult hearts following MI in vivo.	Eulalio et al., 2012

Name	In Vivo/ In Vitro	Summary	Citation
miR302–367	In vivo (mouse)	Postnatal reexpression of miR302–367 reactivated the cell cycle in CMs and induces proliferation by targeting several components of the Hippo signal transduction pathway, resulting in reduced scar formation after experimental MI.	Tian et al., 2015
miR-499	In vitro (mouse P19CL6 cells and neonatal rat ventricular CMs)	miR-499 promotes the proliferation of neonatal CMs via its effect on Sox6 and cyclin D1.	Li et al., 2013
T3 (Triiodothyronine)	In vivo (mouse)	A thyroid hormone surge activates the IGF-1/IGF-1-R/Akt pathway on postnatal day 15 and initiates a brief but intense proliferative burst of predominantly binuclear CMs.	Naqvi et al., 2014