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Regeneration of the heart: from molecular mechanisms to clinical therapeutics

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Abstract

Heart injury such as myocardial infarction leads to cardiomyocyte loss, fibrotic tissue deposition, and scar formation. These changes reduce cardiac contractility, resulting in heart failure, which causes a huge public health burden. Military personnel, compared with civilians, is exposed to more stress, a risk factor for heart diseases, making cardiovascular health management and treatment innovation an important topic for military medicine. So far, medical intervention can slow down cardiovascular disease progression, but not yet induce heart regeneration. In the past decades, studies have focused on mechanisms underlying the regenerative capability of the heart and applicable approaches to reverse heart injury. Insights have emerged from studies in animal models and early clinical trials. Clinical interventions show the potential to reduce scar formation and enhance cardiomyocyte proliferation that counteracts the pathogenesis of heart disease. In this review, we discuss the signaling events controlling the regeneration of heart tissue and summarize current therapeutic approaches to promote heart regeneration after injury.

Key words Heart regeneration, Cardiac disease, Therapeutics, Signaling mechanisms

Background

Cardiovascular disease is the leading cause of death and accounts for approximately 32% of global deaths, resulting in the losses of 17.9 million lives each year[1,2]. Military personnel is significantly more likely to report higher work-related stress than civilians[3,4], contributing to the long-term development of cardiovascular diseases and acute triggering of heart failure[5]. Cardiovascular disease represents the cause of more than 10% of military pilots' groundings[6]. The rate of heart failure among hospitalized veterans reaches as high as 0.5%[7]. These studies highlight the importance of cardiovascular research in military medicine. Despite tremendous efforts and advances in cardiovascular research and therapies, heart failure continues to maintain high mortality and morbidity rates[1,8]. Taking longer life expectancy, higher rates of obesity, diabetes, and modern lifestyle into consideration, epidemiologic studies predicted a 46% increase in heart failure patients by 2030[9,10]. Figure 1 illustrates the standard of care for managing heart failure. Currently, pharmacological treatment can slow down heart failure progression, but it still needs a breakthrough.

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Potential approaches for cardiac regeneration have been tested, including strategies based on *in situ* cellular reprogramming and *de novo* tissue engineering methods. Although promising data have been accumulated, each of these approaches faces challenges. Cardiomyocytes of the adult human heart are terminally differentiated and have virtually no regenerative capacity, making it hard to reboot the proliferation of cardiomyocytes after injuries[11]. Although tissue engineering approaches have developed rapidly owing to the improvement of biomaterials and 3D printing, creating a functional heart *in vitro* remains a great challenge[12]. Stem cell-based therapies attempt to promote heart regeneration by injecting stem cells into patients. However, the survival, anchor, differentiation, and maturation of stem cells at the injured site are hard to control, and thus require further optimization before being ready for clinical practice[13,14]. Recent studies suggest that the substances secreted by stem cells may promote heart regeneration[15,16], initiating the search for drugs that target the molecular signaling pathways induced by these substances. Therefore, further understanding the molecular mechanism controlling heart regeneration will help to facilitate the emergence of new therapies that could restore cardiac function. This review summarizes the molecular signaling pathways for heart regeneration and discusses the progress and challenges of approaches for heart regeneration.

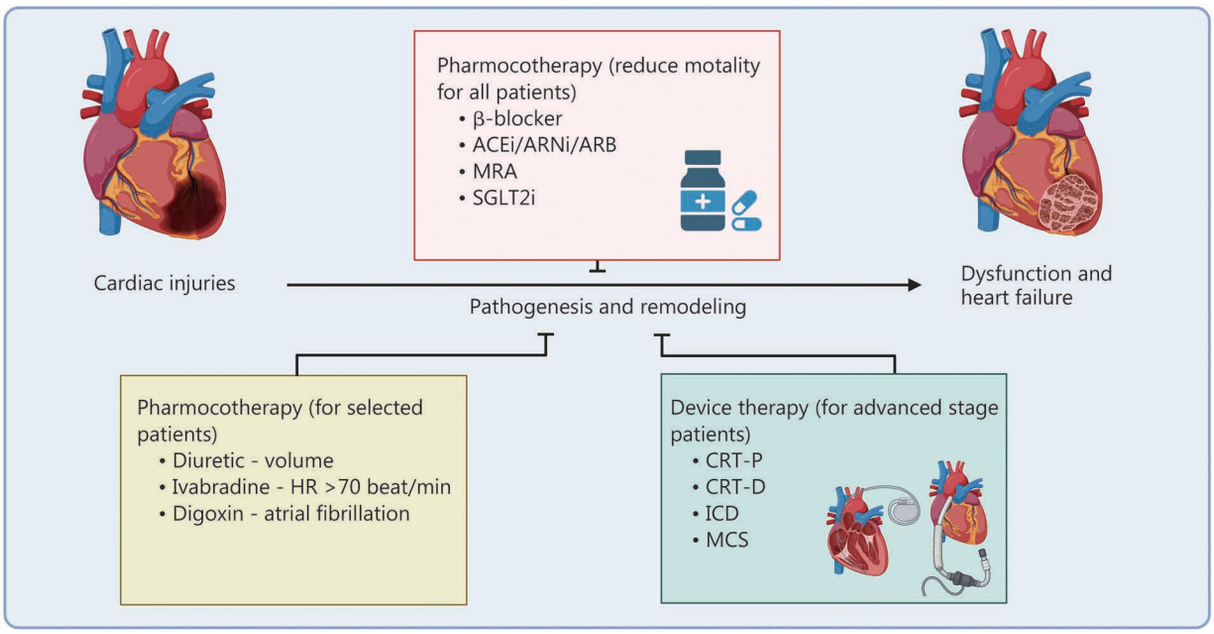

Fig. 1 Standard of care for heart failure.
Pharmacological treatment and medical devices are currently being used to manage the progression of diseases. β-blocker, ACEi, MRA, and SGLT2i are usually used for all patients with heart failure in order to reduce mortality. For selected patients, diuretic, ivabradine, and digoxin might be used. For advanced stage patients, device and surgery would be recommended. ACEi. Angiotensin-converting enzyme inhibitor; ARNi. Angiotensin receptor neprilysin inhibitor; ARB. Angiotensin receptor blocker; MRA. Aldosterone receptor antagonists; SGL2i. Sodium glucose cotransporter 2 inhibitor; HR. Heart rate; CRT-P. Cardiac resynchronization therapy-pacemakers; CRT-D. Cardiac resynchronization therapy-defibrillators; ICD. Implantable cardioverter defibrillator; MCS. Mechanical circulatory support

Role of molecular signalings in heart regeneration

Notch and Notch intracellular domain (NICD) promote cardiomyocyte proliferation and inhibit immune cell infiltration

Heart regeneration was first described in zebrafish 20 years ago by Poss *et al*.[17]. Since this milestone study, the underlying signaling pathways have been extensively studied, as summarized in Fig. 2, first, showing that Notch mediates heart generation[18]. Since then, efforts have been made to understand the signaling events boosting cardiomyocyte proliferation, with the hope of aiding human heart regeneration. Notch signaling plays an important role in regulating endocardium maturation *via* serpine1. Inhibiting or activating Notch both result in impairment of heart regeneration, indicating a dynamic change of Notch activity is crucial[19]. In addition, Notch signaling in the endocardium interacts with cardiomyocytes as an antagonist for Wnt signaling and promotes cardiomyocyte proliferation[20]. Following the initial inflammatory response, the endocardium and epicardium regenerate first to provide the right environment for cardiomyocyte proliferation. For example, the endocardium and epicardium secrete retinoic acid, and the epicardium produces fibronectin of extracellular matrix (ECM)[21,22]. The newly-formed heart muscle is found to populate *via* cardiomyocyte dedifferentiation and proliferation[23]. A study by Gemberling *et al*.[24] demonstrated that neuregulin 1 (Nrg1) is up-regulated after heart injury and serves as a potent inducer of cardiomyocyte proliferation. Notch signaling is also involved in this process, and a remarkable increase in Notch1b and DeltaC expression has been observed[18]. Interestingly, both Notch inhibition and Notch overexpression have been found to inhibit cardiomyocyte proliferation and heart regeneration, suggesting a delicate balance of this pathway is required[25]. Further studies by Pfefferli *et al*.[26] and Gupta *et al*.[27] have distinguished the contribution of different layers of cardiomyocytes during regeneration. Fate mapping with *careg:EGFP* has shown that the primordial cardiac layer incompletely regenerates after cryoinjury and grow restrictively by lateral expansion, while cortical and trabecular layers are primarily responsible for myocardium growth. When overexpressed specifically in cardiomyocytes, Notch also improves cardiac function by reducing the formation of scars[28]. Notch signaling pathway as a potential target for therapeutic approaches has been recently discussed[29]. Functional screening of congenital heart disease risk loci shows that *maml3* mutants can decrease cardiomyocyte proliferation

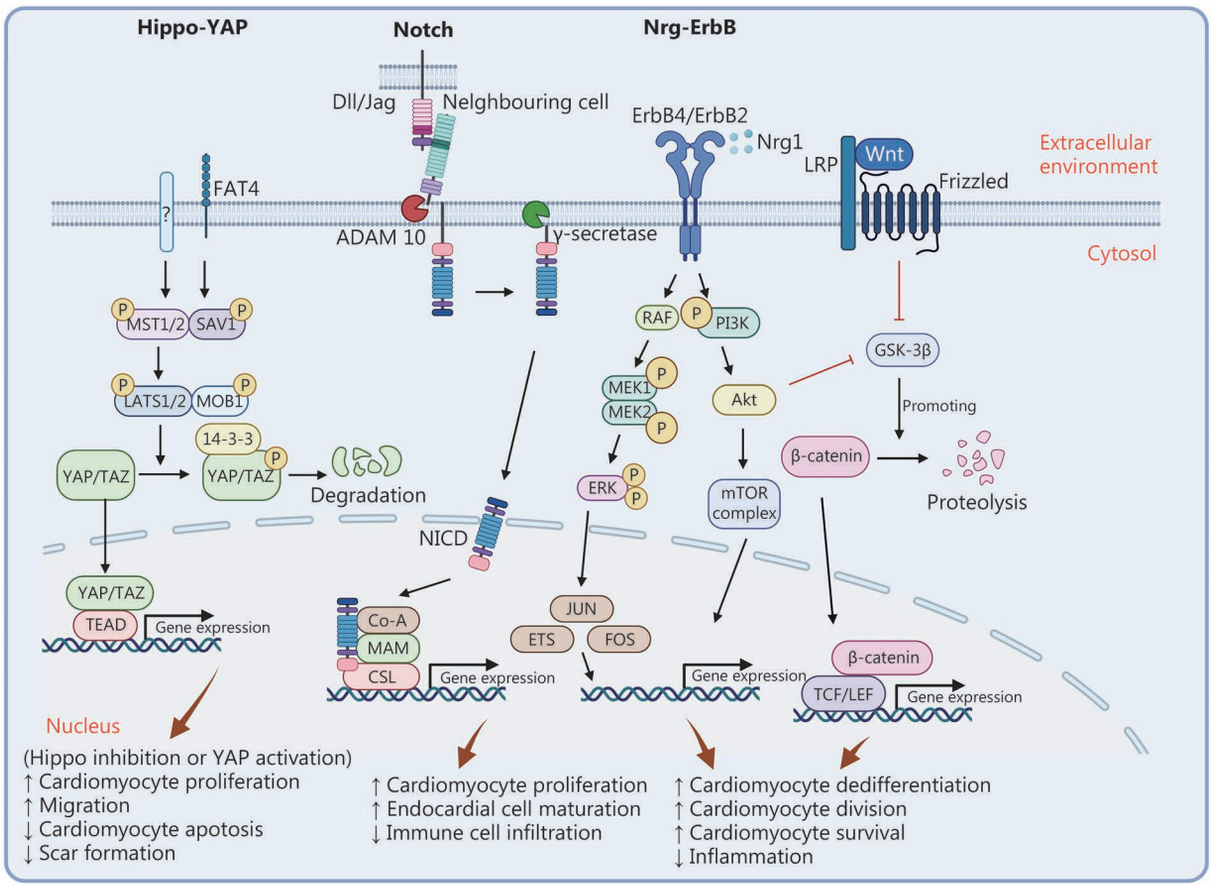

Fig. 2 Signaling pathways in heart repair and regeneration.

Hippo-YAP, Notch and Nrg-ErbB signaling pathways are the major players in regulating heart repair and regeneration after injuries. Hippo-YAP regulates cardiomyocyte proliferation, migration, and apoptosis, thus affecting scar formation after injury. Notch signaling controls cardiomyocyte proliferation, as well as immune cell infiltration and endocardial cell maturation. Nrg-ErbB signaling affects cardiomyocyte dedifferentiation, division, and survival. FAT4. FAT atypical cadherin 4; MST. Macrophage stimulating; SAV1. Salvador family domain-containing protein 1; LATS. Large tumor suppressor kinase; MOB1. MOB kinase activator 1; YAP. Yes-associated protein; TAZ. Tafazzin, phospholipid-lysophospholipid transacylase; TEAD. TEA domain family; ADAM. ADAM metallopeptidase domain; NICD. Notch intracellular domain; MAM. Mastermind; CSL. Citrate synthase like; ErbB2. Erb-B2 receptor tyrosine kinase; RAF. v-raf-leukemia viral oncogene; PI3K. Phosphatidylinositol 3-kinase; MEK1. Mitogen-activated protein kinase kinase 1; ERK. Extracellular signal-regulated kinase; Akt. Protein kinase B; mTOR. Mechanistic target of rapamycin kinase; JUN. Jun proto-oncogene; ETS. ETS transcription factor family; FOS. FBJ osteosarcoma oncogene; LRP. LDL receptor related protein; GSK-3β. Glycogen synthase kinase-3β; TCF. T-cell factor; LEF. Lymphoid enhancer factor

through inhibition of Notch signaling[30], indicating that overexpression of *maml3* may induce cardiomyocyte proliferation by activating Notch.

Hippo and Yes-associated protein (YAP) regulate cardiomyocyte proliferation and scar formation

The Hippo-YAP pathway is highly conserved and plays a pivotal role in cardiomyocyte cell cycle re-entry. Hippo deficiency enhances cardiomyocyte regeneration and heart functional recovery while reducing scar formation after myocardial infarction in adult mice[31,32]. The Hippo-deficient cardiomyocytes express higher levels of proliferative and stress response genes, such as *Park2*[32]. YAP, the inactivated downstream effector of Hippo, is abundant in neonatal heart tissue but not in adult heart tissue. Recent studies found YAP to be a key regulator for cardiac development and regeneration in mice[33-35]. Similar to inhibiting Hippo, activation of YAP results in less scar formation and improved heart function after myocardial infarction at postnatal days 7 and 28 as well as adult stages[35,36]. In Erb-B2 receptor tyrosine kinase (ErbB2)-overexpressed mice, YAP mediated a robust epithelial-mesenchymal transition (EMT)-like regeneration by interacting with the cytoskeleton and altering the mechanical characteristics of the cell[33]. In addition, non-coding RNAs make up a major part of the complex regulatory signaling network. Eulalio *et al.*[37] found that miR-199a and miR-590 can effectively induce cell cycle re-entry of cardiomyocytes

in vitro as well as in neonatal and adult mice. In murine myocardial infarction models, overexpression of miR-199a and miR-590 *via* single-dose injection of synthetic RNA promotes cardiac regeneration and recovery of cardiac function[37,38]. Recently, Gabisonia *et al.*[39] found that, using infarcted pig hearts, miR-199a was shown to facilitate cardiac repair and increase muscle mass and contractility. Follow-up studies on miR-199a have identified potential downstream signaling, such as CD151[40], mechanistic target of rapamycin (mTOR)[41] and Wnt2[42]. Cardiac-specific overexpression of miR-128 in neonatal mice attenuates cardiomyocyte proliferation and functional recovery after myocardial infarction. miR-128 regulates cardiomyocyte cell cycle re-entry *via* SUZ12, a chromatin modifier that targets p27, cyclin E, and CK2[43]. Overexpression of miR-195 (a member of miR-15) leads to reduced proliferation and hypertrophy of cardiomyocytes, while inhibition of the miR-15 family increases cardiomyocyte proliferation after myocardial infarction in adult mice. The downstream target of miR-195 includes cell cycle genes, mitochondrial genes, and inflammatory genes[44]. Similarly, miR-1/-133a is also a negative regulator of cardiomyocyte cell cycle re-entry in the adult heart. Short-term deletion of miR-1/-133a protects against myocardial infarction. However, long-term deficiency leads to heart failure[45]. circNfix, a circular RNA, is up-regulated in the adult hearts of humans and mice. Knocking down circNfix releases suppression on downstream cyclinA2 and cyclinB1 and increases miR-214 activity, leading to enhanced cardiomyocyte proliferation and recovery after injury[46]. miR-152 has been found to be a target of Toll-like receptor 3 (TLR3) and induces cardiomyocyte proliferation by regulating cell cycle proteins downstream of YAP1[47]. Recent study shows that FAM122A, an endogenous inhibitor of protein phosphatase 2A, is a novel regulator in the mesendodermal specification and cardiac differentiation *via* Hippo and Wnt signaling pathways[48]. In the first step, RNA-binding protein LIN28a stimulates the formation of new cardiomyocytes and prevents cardiomyocyte apoptosis[49]. Activation of YAP promotes progenitor regeneration by triggering LIN28a transcription[50].

To date, little is understood about the removal of the scar and the functional integration of regenerated cardiomyocytes. The collagenolytic activity was detected in the injured region from day 14 to day 30. In the same period, expressions of matrix metalloproteins (MMPs), such as MMP-2 and MMP-14a, are up-regulated, suggesting a potential role for them in scar removal[51]. Expression of miR-101a is inhibited after the onset of injury but up-regulated from day 7 to day 14. Suppression of miR-101a promotes cardiomyocyte proliferation but inhibits scar removal. Depletion of the downstream target gene *Fosab* rescued the scar-clearing defect of miR-101a inhibition, demonstrating that miR-101a regulates scar removal *via Fosab*[52]. Scar formation is regulated by YAP signaling, and macrophages directly produce collagen to make up the scar[53,54]. Deletion of YAP from zebrafish does not affect the proliferation of cardiomyocytes but leads to larger injuries, showing that initial scar formation is important to control the damage[53]. In zebrafish, fibrosis does not preclude scar-free regeneration[55,56].

ErbB/PI3K/ERK and Wnt/β-catenin control cardiomyocyte proliferation, dedifferentiation, and inflammation

The Nrg1/ErbB has been recognized as a potential signaling pathway involved in the heart regeneration program. Nrg1 was initially proposed for its potential relevance to mitogenic effects in mammalian cardiomyocytes and further was proved in the post-injured zebrafish heart by Gemberling *et al.*[24], which provided the foundation for mouse experiments and clinical trials. In adult mice, injection of Nrg1 induces cell cycle re-entry and cardiomyocyte division. Inactivation of the tyrosine receptor ErbB4 for Nrg1 reduces cardiomyocyte proliferation, while stimulation of ErbB4 enhances it[57]. The deletion of another co-receptor for Nrg1, ErbB2, also shows its importance for cardiomyocyte proliferation in neonatal mice. Constitutive activation of ErbB2 in both neonatal and adult mice leads to cardiomyocyte proliferation and dedifferentiation *via* extracellular signal-regulated kinase (ERK), protein kinase B (Akt) and glycogen synthase kinase-3β (GSK-3β)/β-catenin downstream signaling. Notably, transient activation of ErbB2 promotes regeneration after myocardial infarction in mice[58].

The initial inflammatory response is required for complete regenerative capacity. Anti-inflammatory treatment reduces cardiomyocyte proliferation and impairs the vascularization of newly-formed tissue, resulting in an inability to clear the fibrotic deposition[59]. In contrast, the immune cell is not required for cardiomyocyte mitotic activity under normal conditions[59]. Fang *et al.*[60] have found that inflammatory cytokines promote cardiomyocyte proliferation *via* activating JAK1/STAT3 signaling. Inhibiting this signaling by expressing a dominant negative form of STAT3 leads to a reduction in cardiomyocyte proliferation. MAPK/ERK acts as a critical signaling for vertebrate tissue regeneration; its potential roles in tissue engineering and regenerative medicine have been emphasized[61]. Kynurenine stimulates cardiomyocyte proliferation by activating the cytoplasmic aryl hydrocarbon receptor-SRC-YAP/ERK pathway; it also stimulates cardiac angiogenesis by facilitating aryl hydrocarbon receptor nuclear translocation and increasing vascular endothelial growth factor

A (VEGF-A) expression[62]. Dual-specificity phosphatase 6 (DUSP6), which can dephosphorylate ERK1/2, is a regenerative repressor during zebrafish heart regeneration[63]. Deletion of *Dusp6* in mice improves cardiac outcomes by reducing neutrophil-mediated myocardial damage induced by myocardial infarction-caused inflammation[64]. Furthermore, a DUSP6 inhibitor has been tested in myocardial infarction rats, showing that it improves heart function and suppresses inflammatory cardiac remodeling[65]. In addition, the cardiac-derived ECM may provide an ideal scaffold for heart tissue engineering[66], and nuclear pore numbers are decreased during cardiomyocyte maturation, and this reduces nuclear responses to activation of MAPK induced by extracellular signals[67]. Activation of Nrf1, a stress-responsive transcription factor is seen in regenerating cardiomyocytes. *Nrf1* overexpression can protect the heart from ischemic injury, while deletion inhibits neonatal heart regeneration by affecting proteasome and redox balance[28]. The role of Wnt in promoting cardiomyocyte differentiation has been further investigated, showing that it may provide a powerful tool for stem cell-based regeneration therapy[68]. These studies suggest that the molecular events initiated by extracellular signals may have therapeutic benefits for heart regeneration.

Approaches and challenges for heart regeneration

The fate mapping experiments in mice have shown that new cardiomyocytes originate from pre-existing ones, during homeostasis[69], after injury in adults[69,70], and during neonatal heart regeneration[71]. In addition, using a transgenic line of hypoxia-inducible factor-1α (HIF-1α), Kimura *et al.*[70] showed that hypoxic cardiomyocytes exhibit characteristics of neonatal heart cells and contribute mostly to cardiomyocyte formation in adults. Despite these results, many efforts have been focused on the c-Kit$^+$ progenitor cells from the bone marrow[72], which were later shown to play a negligible role in heart regeneration[73]. Using the Cre/lox system and a reporter line, endogenous c-Kit$^+$ cells are found to generate cardiomyocytes at a percentage less than 0.03. Although c-Kit$^+$ cells contribute to the revascularization of cardiac endothelial cells, their role in myocardium regeneration is insignificant.

In order to develop new therapies, recent studies have worked on understanding the regulatory role of nonmuscle cells, such as immune cells, endothelial cells, and cardiac fibroblasts. In neonatal mice, CD4$^+$ regulatory T cells (Tregs) are necessary for cardiac regeneration. Depletion of Tregs inhibits cardiomyocyte proliferation and induces fibrosis, whereas adoptive transfer of Tregs rescues this phenotype[74]. Interestingly, ablation of CD4$^+$ Tregs in mice at postnatal day 8 promotes heart regeneration after resection[75], suggesting the role of immune cells might differ by stages. Endothelial cells support heart regeneration by reassembling arteries, which serve as a scaffold for cardiomyocyte repopulation and also reperfuse the ischemic tissues[76,77]. Endothelial cell migration is induced by the CXCL12-CXCR4 signaling pathway. Genetic inhibition of this signaling leads to formation of larger scars and the reduction of cardiomyocyte proliferation after myocardial infarction[76]. Consistent with this, inhibition of revascularization in zebrafish with dominant negative VEGF-A also hindered regeneration, suggesting that endothelial cells are actively engaged in cardiomyocyte proliferation[78]. Cardiac fibroblasts deposit ECM and their number increases during development and diseases, such as heart failure[79]. Transcriptomic analysis showed different gene expression profiles between fetal and adult fibroblasts of humans, suggesting fibroblasts might be potential contributors to embryonic heart regeneration[80]. However, ablating activated fibroblasts in mice has a protective effect after acute injuries[81], which contradicts its vital function in promoting heart regeneration of zebrafish[82]. This could potentially be explained by the existence of different sub-clusters of fibroblasts in the heart, but further studies are still needed[83]. In summary, modulating immune cells, endothelial cells, and fibroblasts after injury may promote cardiac regeneration and lead to further mitigation of disease.

The regenerative ability of the mammalian heart is lost during development. In humans, the scar-free repair of the heart is feasible, but only at early developmental stages[84,85]. A case report of a newborn child by Haubner *et al.*[86] showed strong regeneration ability after severe myocardial infarction and tissue damage. The cardiac function of this 1-year-old child recovered a few weeks after the initial injury. Similar responses have been seen in other cases by Cesna *et al.*[87], Deutsch *et al.*[88], and Farooqi *et al.*[89], leading to the hope of repairing a damaged adult heart by reactivating regenerative processes that are present during the neonatal stage. Similar to humans, mice lose the capacity for heart regeneration during the early postnatal stage from postnatal day 1 to day 7[84]. A well-designed study by Drenckhahn *et al.*[90] showed that embryonic cardiomyocytes are able to re-enter the cell cycle and proliferate to form heart muscles. In this study, the X-linked gene *Hccs* was deleted specifically in the heart muscle; this deletion is lethal for the cell. In heterozygous females (half of the cardiomyocytes were normal due to random X inactivation), the mutant cells contributed to less than 10% of tissue volume, showing that the normal

cardiomyocytes are able to regenerate about 50% loss of cardiomyocytes at embryonic stage[90]. By removing 10% of the ventricle from mice at various ages, the time windows of regeneration are characterized[71]. The murine heart can regenerate at postnatal day 1 after surgical resection with minimal scar or hypertrophy[91]. This regenerative ability is continuously lost until it ceases at postnatal day 7. In support of this conclusion, similar results have been observed in many other injury models by Haubner *et al.*[92] and Porrello *et al.*[44] although the collagen scar has been observed when resecting a larger part (20%) of the ventricle[93]. A study by Porrello *et al.*[44] using left anterior descending artery (LAD) ligation-induced injury showed that the heart regenerates within 3 weeks after extensive necrosis. This study compared changes in gene expression after injury between postnatal days 1, 3, and 10. Many genes regulating mitosis, cell division, cell cycle, and ECM synthesis have been identified, including *NPPA* (atrial natriuretic factor), *Nanog* (stem cell marker), and *HIF3A* (hypoxia-inducible factor-3α gene)[92]. Further study by Darehzereshki *et al.*[91] with cryoinjury models has revealed different modes of repair after different types of injury. Neonatal hearts are able to regenerate after non-transmural cryoinjury but not after transmural injury and differential plasminogen activator inhibitor-1 (PAI-1) expression could be a potential explanation. Konfino *et al.*[94] found that both neonatal and adult mice respond differently to LAD-induced myocardial infarction and resection. The adult heart forms a thin scar after myocardial infarction, whereas apical resection leads to the occurrence of a hemorrhagic scar. Together, these findings suggest that different treatments should be developed to administer to specific injuries.

The limitation of this model is the lack of cell death, inflammation, and debris clearance steps during the healing process[95]. Cryoinjury is one of the most commonly used methods, in which a precooled metal is used to freeze part of the ventricle[55,56]. Although cardiac tissue loss is similar to the resection model, it takes much longer, around 130 d, to regenerate the heart after cryoinjury[56]. Genetic models of cardiomyocyte death have also been used to study heart regeneration in zebrafish. Wang *et al.*[96] ablated cardiomyocyte with the expression of cytotoxic diphtheria toxin A chain, induced by cell-specific cyclization recombination enzyme (Cre). This method induces around 60% loss of cardiomyocytes while leaving the endocardium and epicardium intact, which resembles cardiomyopathy in human patients[97]. Heart function and tissue are restored in around 30 d, which could be attributed to the importance of epicardium in heart regeneration[98].

Using these injury models, the cellular processes of heart regeneration have been better characterized and a signaling network of genes was identified to be crucial for scar-free regeneration. The regenerative process can be separated into four major stages: 1) the acute reaction to injury, including recruitment of immune cells and deposition of fibrotic tissues; 2) the endocardium and epicardium regenerate in order to support the myocardium; 3) the myocardium is regenerated *via* proliferation, and 4) the functional integration of newly generated cardiomyocytes, scar removal, and inflammation resolution[95].

Transplantation of progenitor-derived cells and stem cells

Cell transplantation to repair the injured heart has been started for more than a decade. Intracoronary administration of bone marrow-derived progenitor cells can improve the recovery of left ventricular contractile function in patients with acute myocardial infarction[99]. However, studies with double-blind randomized designs show that injection of bone marrow mononuclear cells fails to improve the left ventricular contractile function[100-102]. The randomized placebo-controlled study of myoblast transplantation also shows that myoblast injections are unable to improve echocardiographic heart function[103]. Adverse effects such as arrhythmias are always problematic, as skeletal myoblasts are not able to conduct electromechanical signals as cardiomyocytes[104]. Therefore, efficient treatment may be cell-specific and achieved by transplantation of progenitor-derived cells. Recent study has graded mesoderm assembly controls cell fate and morphogenesis of the early mammalian heart[105].

Another approach is to induce the differentiation of cardiomyocytes *in vitro* using embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Both cell types are able to succeed *in vitro* to produce cardiomyocyte-like cells[106,107]. Convincing evidence shows that transplantation of ESC-derived cardiomyocytes improves heart function by integrating with pre-existing cardiomyocytes to transduce electromechanical signals[108,109]. Although transplantation of human ESC-derived cardiomyocytes can regenerate the infarcted pig heart, it induces ventricular tachyarrhythmias[110]. There have been few clinical trials in humans given the ethical challenges of ESCs as well as concerns about side effects. One trial shows some positive results, but with an overall low engraft rate and lack of careful characterization of the control group[111]. Similarly, another trial shows that transplantation of iPSC-derived cardiomyocytes improves ventricular contractility and promotes heart regeneration, but has low engraftment and survival rate of cardiomyocytes, and induces complications

such as tachycardia[112,113].

The POSEIDON study shows that bone marrow-derived mesenchymal stem cells (MSCs) may have cardiogenic potential and improve the functional capacity of the heart[114]. However, the conclusion is hindered by the lack of a placebo control group and a small patient cohort of 30. However, a randomized double-blind trial shows that bone marrow-derived mesenchymal stromal cells produce a moderate improvement in left ventricular ejection fraction (LVEF) and stroke volume of ischemic heart[115]. Similar results have been reported in trials using MSCs derived from different sources, such as the umbilical cord-mesenchymal stem cell (UC-MSC)[116]. The Congestive Heart Failure Cardiopoietic Regenerative Therapy (CHART-1) trial demonstrated that MSC injection is overall safe[117] and has long-term benefits in patients with significant left ventricular enlargement[118,119]. The recent CONCERT-HF trial shows that MSC in combination with c-Kit positive cells (CPCs) can significantly reduce heart failure-related major adverse cardiac events (HF-MACE). However, no improvement in left ventricular function or reduction of scar size can be achieved, requiring further elucidation of the underlying mechanism[120,121]. Other clinical trials show that MSC injection fails to produce functional improvement of the heart[122,123]. Although MSCs can differentiate into cardiomyocytes *in vitro*[124], MSC-derived endothelial cells are the main contributor to heart regeneration in animal model[125]. A randomized double-blind multi-center trial TEAM-AMI shows that the efficacy of MSC injection is highly dependent on the microenvironment[126], supporting that the clinical benefits are mainly mediated by indirect effects instead of by generating new cardiomyocytes[123]. Vagnozzi *et al.*[127] showed that intracardiac injection of killed stem cells or use of chemical inducers for immune response produced similar results as live adult stem cell. All these treatments induce a regional accumulation of $CCR2^+$ and $CX3CR1^+$ macrophages, which affect fibroblasts and the ECM at the injury site. A series of animal studies by Bolli *et al.*[128] demonstrated that transplanted cells cannotengraft into the myocardium nor differentiate to cardiomyocytes, although improved cardiac function was observed. This dissociation of therapeutic improvement with engrafting rate has been seen among MSCs, ESCs, and CPCs treatment, independent of delivery method and preconditions[129]. These new findings suggest that the benefits from stem cell injection are mainly due to secreted factors instead of cell replenishment. Therefore, understanding the molecular signaling induced by factors secreted by stem cells becomes more important for treatment of heart injury. Recent studies show that endoderm-derived islet1-expressing cells can differentiate into endothelial cells to function as hematopoietic stem and progenitor cells[130], which may serve as an alternative approach for stem cell transplant; in addition, human- or animal-derived decellularized heart patches have been used *in vivo* and *in vitro* studies to promote the regeneration of heart tissue[131]. However, due to the complexity of cardiac tissue engineering, significant hard work must be done before the approaches can be clinically used.

Currently, a growing number of clinical trials[130] (see Bolli *et al.*[129] for a comprehensive list of trials) and Meta-analyses [132] have greatly expanded the knowledge and potential choices of cell sources and interventions for heart disease, such as IMMNC-HF with bone marrow mononuclear cell[133]; LAPiS (NCT04945018), HEAL-CHF (NCT03763136) and NCT05223894 with human iPSC derived cardiomyocytes; NCT05147766 with UC-MSCs; NCT03797092 with adipose-derived stromal cell; and BioVAT-HF (NCT04396899) with engineered human myocardium. DREAM-HF, a phase III clinical trial, recruited 565 patients and upon completion will provide evidence in analyzing the efficiency of MSC injection as a heart failure treatment[14,134,135]. Recent study showed that human mesenchymal stromal cells and endothelial colony-forming cells reduce cardiomyocyte apoptosis, scar size, and adverse cardiac remodeling, compared to vehicle, in a pre-clinical model of acute myocardial infarction[136]. Human ESC-derived endothelial cells also attenuate cardiac remodeling in a mouse myocardial infarction model[137]. Besides cardiomyocytes, cardiac interstitial cells also play crucial roles during cardiac regeneration[138], which opens another avenue to improve heart regeneration. These studies provide useful information for cell therapy approach to treat cardiac injury in the future.

Inducing proliferation of existing cardiomyocytes

The safest and least immunogenic option for cardiac regeneration is using pre-existing cardiomyocytes, although human cardiomyocytes are well-known for being non-proliferative[85]. There is evidence supporting that cardiomyocytes self-renew at a slow but steady speed[69], and previous mechanistic studies in mice and zebrafish have provided clues for potential therapeutic targets. Combined expression of cell cycle-related genes, *Cdk1*, *Ccnb*, *Cdk4*, and *Ccnd* induces post-mitotic cell proliferation and improves ventricular function after myocardial infarction[139]. As discussed earlier, the Hippo-YAP pathway is a promising target for promoting cardiomyocyte proliferation. Adeno-associated virus (AAV)-based genetic knockdown of Hippo pathway gene

Sav in pig models has been shown to increase the renewal rate of cardiomyocytes after myocardial infarction and improve LVEF[140]. No arrythmia, tumor formation, or mortality has occurred after treatment, making this a promising approach to advancing clinical trials.

Another potential target is Myc, a transcription factor involved in cell replication, differentiation, metabolism, and apoptosis[141]. Four-hour acute activation of Myc signaling in juvenile mice leads to a marked proliferative response *in vivo*[142]. Mechanistically, this effect is mediated by positive transcription elongation factor b (P-TEFb), which consists of CDK9 and cyclinT1. Furthermore, a transient cardiomyocyte-specific expression of Myc, SRY-box transcription factor 2 (SOX2), OCT4 (named POU5F1; POU domain, class 5, transcription factor 1), and KLF transcription factor 4 (KLF4) can induce dedifferentiation of adult cardiomyocytes characterized by a gene expression profile resembling that of fetal cells. This allows the reprogrammed cardiomyocytes to reenter the cell cycle and divide into more cardiomyocytes, leading to improved cardiac function after myocardial infarction[143]. Prolonged expression of these 4 factors resulted in tumor formation and lethality in mice, however, urging the need for more in-depth studies to avoid potential safety issues.

The Nrg1 has shown its mitogenic effect in pre-existing cardiomyocytes (mentioned in section "ErbB/PI3K/ERK and Wnt/β-catenin control cardiomyocyte proliferation, dedifferentiation, and inflammation"). Furthermore, Polizzotti *et al.*[144] show that recombinant neuregulin 1 (rNRG1) induces the proliferation of cardiomyocytes both in mice and in isolated human myocardium, which opened the therapeutic window and prompted clinical trials. A double-blind, placebo-controlled clinical trial of neuregulin 1β3 (cimaglermin alfa) shows sustained improvements in LVEF[145]. Another clinical trial shows that recombinant human neuregulin 1 (rhNRG1) can increase LVEF and decrease end-diastolic volume (EDV) and end-systolic volume (ESV) in chronic heart failure patients. However, these results were statistically indistinguishable from the placebo, and it remains unclear if this treatment improves heart function by inducing regeneration[146]. Overall, there is active research underway to develop and optimize therapies using identified gene targets and to explore new targets, i.e., Hoxb13 by Nguyen *et al.*[147], Meis1 by Mahmoud *et al.*[148], and miR-199a by Eulalio *et al.*[37] and Gabisonia *et al.*[39].

Reprogramming non-muscle cells into cardiomyocytes

Reprogramming other cells of the heart, such as fibroblasts, into cardiomyocytes, is another way to achieve the challenging task of repairing the heart. As a large cell population of the heart[149], fibroblasts are the first responders after cardiac injuries, thus making them an ideal source of cardiomyocytes. Forced expression of cardiac transcription factor combinations, such as GATA binding protein 4 (GATA4), myocyte enhancer factor 2C (MEF2C), and T-box transcription factor 5 (TBX5) (GMT cocktail)[150]; or GATA4, heart and neural crest derivatives expressed transcript 2 (HAND2), MEF2C and TBX5 (GHMT)[151], can successfully transform fibroblasts into cardiomyocytes *in vitro*. Bypassing the iPSC stage, this approach reprograms fibroblasts directly into contractile cardiomyocytes that express typical cardiomyocyte markers. *In vivo* expression of GHMT using retroviral infection in mice showed that reprogramed cells can form cardiomyocytes and conduct electromechanical signals after myocardial infarction induced by LAD ligation[151]. Many genes and signaling pathways involved in heart regeneration also modulate reprogramming of fibroblast into cardiomyocytes, including Notch signaling[152], zinc finger transcription factor 281 (ZNF281; regulating inflammation)[153], fibroblast growth factor (FGF) and VEGF[154], Akt1/protein kinase B[155], Bmi1 (epigenetic factor)[156], and chemical factors[157]. Recently, Wang *et al.*[158] found that autophagic factor Beclin1 negatively regulates fibroblast reprogramming in an autophagy-independent manner, and that Beclin1 haploinsufficiency in mice promotes reprogramming and reduces scar size after myocardial infarction. In addition, a combination of miRNAs, miR-1, -133, -208, and -499 have also been found to induce cardiomyocytes from fibroblasts both *in vitro* and *in vivo*[159,160], providing alternative targets for fibroblast reprogramming. Alternatively, Lalit *et al.*[161] showed that mesoderm posterior bHLH transcription factor 1 (MESP1), GATA4, TBX5, NK2 homeobox 5 (NKX2-5), and BAF60C (SMARCD3, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily D, member 3) expressed in fibroblasts produce a progenitor population that gives rise to cardiomyocytes, endothelial cells, and mural cells in myocardial infarction mice models. Recent study also suggests that the cardiac gene TBX20 (T-box transcription factor 20) enhances myocardial reprogramming induced by the MGT133 reprogramming cocktail (MEF2C, GATA4, TBX5, and miR-133)[162]. In summary, transcription factor combinations play an important role in transforming fibroblasts into cardiomyocytes in mice.

Despite the success in mice, human fibroblasts are more resistant to both the transcription factor and miRNA combination-induced reprogramming and have shown overall inadequate efficacy to produce cardiomyocytes.

Furthermore, the induced cardiomyocytes mostly lack contractility[163,164]. Follow-up studies discovered that the reprogramming process of human fibroblasts requires the addition of other factors, such as MESP1 and myocardin (MYOCD)[165,166], ZFPM2 (zinc finger protein, FOG family member 2)[166], V-Ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) and MESP1[167]. More efforts are still needed to understand the molecular mechanism and the heterogeneity[168] of induced cardiomyocytes and improve the efficacy of this approach before clinical application. Nevertheless, studies using mouse models have reached a new level by using a novel Tcf21iCre/reporter/MGTH2A transgenic mouse system showing that cardiac reprogramming can repair myocardial infarction[169]. However, whether it is safe and efficacy for patients remains to be validated.

Non-cell-based approaches

Although still in the early stages, approaches that are not based on cells have the great potential as they bypass the difficulties related to low engraft rates, unclear mechanism, and ethical and safety problems. Study by Puente *et al.*[170] in postnatal mice found that oxidative stress induces cell cycle arrest, thus contributing to the loss of heart regenerative ability. Based on this finding, Nakada *et al.*[171] designed experiments where mice were exposed to hypoxia for a week after myocardial infarction. This treatment triggers a robust regenerative response and improves left ventricular systolic response. Fate-mapping showed that pre-existing cardiomyocytes proliferate to form myocardium, making it an intriguing idea to treat patients with gradual systemic hypoxia.

Secreted factors, such as growth factors VEGF-A, FGF-2, Nrg1, and thymosin β4, protect against myocardial injuries in animal models[172,173]. However, this effect has not been seen in clinical trials with both VEGF-A and FGF-2[174,175]. One explanation for this might be that the delivery method cannot ensure a high bioavailability, as a better recovery is achieved by using synthetic mRNA to express VEGF-A in mice[176]. Recent study showed that VEGF-A-induced angiogenic sprouting can be attenuated by siRNA knockdown or CRISPR/Cas9 knockout of LINC00607[177]. VEGF mRNA has been administrated to patients *via* direct intramyocardial injection, showing that it may be safe for introducing genetic material to the cardiac muscle[178]. Nrg1 sustains the epicardial-mediated cardiac regeneration capacity of neonatal heart explants[179]. Oxytocin also activates epicardial cells and promotes heart regeneration after cardiac injury[180]. Daily administration of thymosin β4, a peptide known to restore vascularization of the epicardium[181], gives mice the capability of producing new cardiomyocytes and improves recovery after myocardial infarction[182,183]. These studies have been confirmed by a recent report showing that thymosin β4 and also prothymosin α promote cardiac regeneration in mice[184]. Exosomes are small extracellular vesicles containing different cargoes like protein, RNA and lipids[185]. Exosomes secreted by iPSC or cardiac progenitor populations promote cardiac functional recovery in animal models[186,187]. Furthermore, mechanistic studies by Cai *et al.*[188] and Zhou *et al.*[189] showed that the epicardium, similar to stromal stem cell, plays an important role in heart regeneration by both serving as a source for cardiomyocytes, and most importantly, by providing the required paracrine factors[190]. A proteomic study by Arrell *et al.*[191] comparing chronic infarction models with and without human stem cell treatment identified 283 and 450 altered proteins, respectively. This finding could provide a roadmap to future therapeutics using secreted factors. Owing to the advancement of the biomedical engineering field, new methods are being developed to efficiently deliver these factors, including exosomes[192], cardiac patches[193], and bioactive hydrogel[194]. For example, a recent report shows that cardiac tissue regeneration can be induced by the delivery of miR-126 and miR-146a *via* exosomes[195]. Recent studies show that cardiogel-loaded chitosan patches or injectable hydrogels containing anti-apoptotic, anti-inflammatory, and pro-angiogenic agents may have therapeutic benefits for heart injury[196,197]. Together, the precise delivery of factors promoting myocardial proliferation and inhibiting apoptosis and inflammation has the potential to enable heart regeneration *in situ*.

Together, these findings provide exciting new directions for regenerative therapeutics for human heart disease. Notably, there are several barriers that need to be removed before translating these findings to clinical practice, such as the variability between species and the insufficient reproduction of results[198]. By using quantitative measurement, human-animal chimeras[199], large-animal models and platforms, i.e., CIBERCV Cardioprotection Large Animal Platform (CIBER-CLAP)[198,200], standardized protocols and quality-control infrastructure[201], future preclinical studies are anticipated to yield positive clinical results.

Conclusions and perspectives

In summary, active research in the field has revealed common molecular mechanisms for heart regeneration and potential new targets for therapies. These potential gene targets function to regulate immune response, cardiac fibroblast activation,

epicardium recovery, and cardiomyocyte proliferation after injuries. Inspired by these findings, current trials focus on inducing heart regenerative ability by cell-based approaches, including progenitor cell transplantation, inducing cardiomyocyte proliferation, and direct reprogramming. Other ongoing therapeutic explorations involve non-cell-based approaches, such as secreted factors and exosomes. In addition, the contribution of non-cardiomyocytes, such as endothelial cells and the epicardium has been actively studied. Figure 3 illustrates current approaches for heart regeneration. With studies for genetics and genomics developed gradually, gene editing technology, especially CRISPR/Cas9, has made continuous breakthroughs, which opens up a new way to manipulate the genome *in vitro* and *in vivo*, and also provides an unprecedented opportunity to explore the application of gene editing in cardiovascular diseases[202,203]. iPSCs are increasingly being used as substitutes or supplements for animal models of cardiovascular disease[204]. Jiang *et al.*[205] have found that fibroblasts could be reprogrammed into cardiovascular progenitor cells using transgenic methods, which are called CRISPR-induced cardiovascular progenitor cells (ciCPCs). The implanted ciCPCs differentiate into cardiovascular cells *in vivo*, which significantly improve myocardial systolic function and the formation of scars, and provide a new source of cells for myocardial regeneration. With the development of artificial intelligence, Theodoris *et al.*[206] recently developed a machine learning approach to identify small molecules, which correct gene networks

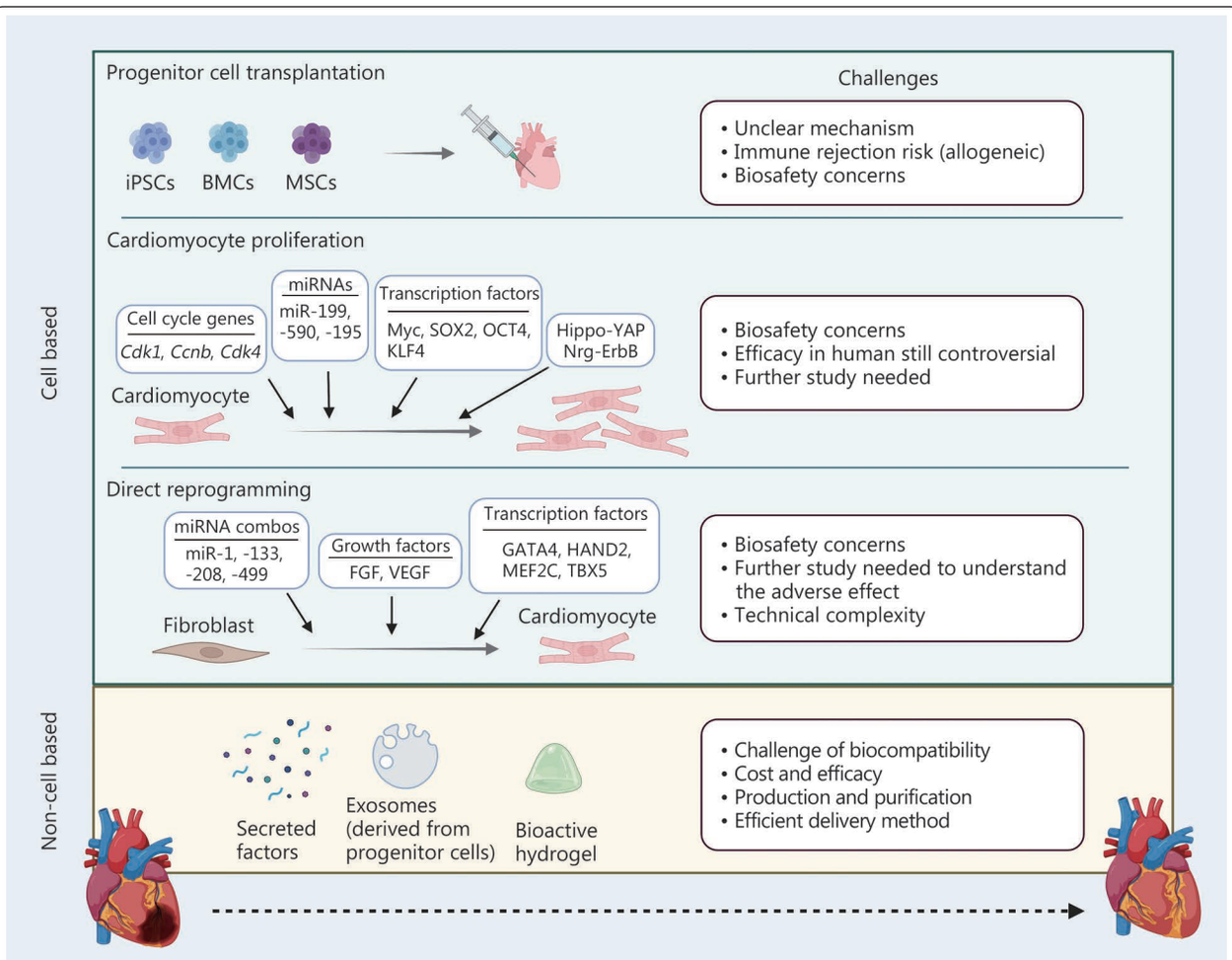

Fig. 3 Current approaches for heart regeneration.
Current attempts at heart regenerative therapies include cell based and non-cell based approaches. Each of these approaches has its own advantages and faces different challenges. iPSC. Induced pluripotent stem cell; BMC. Bone marrow cell; MSC. Mesenchymal stem cell; Cdk1. Cyclin-dependent kinase 1; Ccnb. Cyclin B; SOX2. SRY-box transcription factor 2; OCT4. POU domain, class 5, transcription factor 1; KLF4. KLF transcription factor 4; YAP. Yes-associated protein; Nrg. Neuregulin; FGF. Fibroblast growth factor; VEGF. Vascular endothelial growth factor; GATA4. GATA binding protein 4; HAND2. Heart and neural crest derivatives expressed transcript 2; MEF2C. Myocyte enhancer factor 2C; TBX5. T-box transcription factor 5

dysregulated in iPSC broadly. This approach could prevent and treat specific cardiovascular diseases in a mouse model. This study points to human-machine learning, network analysis, and iPSC technology to make this strategy feasible and potentially represent an effective path for drug discovery[206]. In addition, Lin *et al*.[207] demonstrated that multiplexed CRISPRi screening combined with machine learning confers functional robustness to gene expression. The prediction of synergistic enhancers by machine learning provides an effective strategy for identifying pairs of noncoding variants associated with disease-causing genes beyond the analysis of genome-wide association studies[207]. There's a reasonable prospect that gene editing and artificial intelligence will also bring breakthroughs in heart regeneration in the future. These attempts generated promising results and could be further optimized and then tested in larger populations. Cre recombinase microinjection will help researchers identify the cell progenitors and gene networks involved in organ development[208]. A variety of tissues and organs including hearts have been produced *via* 3D bio-printing, which serves as *in vitro* models for pharmacokinetics and drug screening[209]. Although it is not promised, 3D bio-printing may be used for repairing, or even replacing, an injured heart in the future. We believe that the endeavors in fighting against heart injury will finally lead to a breakthrough for adult heart regeneration.

Abbreviations

AAV: Adeno-associated virus; Akt: Protein kinase B; BAF60C: SMARCD3, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3; BMCs: Bone marrow cells; CHART-1: Congestive Heart Failure Cardiopoietic Regenerative Therapy; CIBER-CLAP: CIBERCV Cardioprotection Large Animal Platform; ciCPCs: CRISPR-induced cardiovascular progenitor cells; CPCs: C-Kit positive cells; ECM: Extracellular matrix; EDV: End-diastolic volume; EMT: Epithelial-mesenchymal transition; ErbB2: Erb-B2 receptor tyrosine kinase; ERK: Extracellular signal-regulated kinase; ESCs: Embryonic stem cells; ESV: End-systolic volume; FGF: Fibroblast growth factor; GATA4: GATA binding protein 4; GSK-3β: Glycogen synthase kinase-3β; HAND2: Heart and neural crest derivatives expressed transcript 2; HF-MACE: Heart failure-related major adverse cardiac events; HIF3A: Hypoxia-inducible factor-3α gene; HIF-1α: Hypoxia-inducible factor-1α; iPSCs: Induced pluripotent stem cells; KLF4: KLF transcription factor 4; LAD: Left anterior descending artery; LVEF: Left ventricular ejection fraction; MEF2C: Myocyte enhancer factor 2C; MESP1: Mesoderm posterior bHLH transcription factor 1; MSC: Mesenchymal stem cell; MYOCD: Myocardin; mTOR: Mechanistic target of rapamycin kinase; NICD: Notch intracellular domain; NKX2-5: NK2 homeobox 5; Nrg1: Neuregulin 1; OCT4: POU domain, class 5, transcription factor 1; P-TEFb: Positive transcription elongation factor b; rhNRG1: Recombinant human neuregulin 1; rNrg1: Recombinant neuregulin 1; SOX2: SRY-box transcription factor 2; TBX5: T-box transcription factor 5; Tregs: Regulatory T cells; TLR3: Toll-like receptor 3; UC-MSC: Umbilical cord-mesenchymal stem cell; VEGF-A: Vascular endothelial growth factor A; YAP: Yes-associated protein; ZFPM2: Zinc finger protein, FOG family member 2; ZNF281: Zinc finger transcription factor 281.

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Author contributions

YJZ designed the framework for this review; QYG wrote the manuscript; JQY and XXF helped to further articulate the logics of this manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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