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The cell-autonomous and non–cell-autonomous roles of the Hippo pathway in heart regeneration

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Abstract

Cardiomyocytes are differentiated heart muscle cells with minimal self-renewal ability. Thus, loss of cardiomyocytes from cardiovascular disease and injury cannot be effectively replenished. Recent studies in animal models have indicated that induction of endogenous cardiomyocyte proliferation is essential for cardiac renewal and that inhibiting the Hippo signaling pathway can stimulate cardiomyocyte proliferation and heart regeneration. Increasing evidence has suggested that cardiomyocyte proliferation requires a permissive microenvironment that consists of multiple cell types. In this review, we summarize recent studies that highlight how the Hippo pathway regulates heart regeneration through cell-autonomous and non–cell-autonomous mechanisms. We also discuss recent translational studies in large animal models that demonstrate the therapeutic potential of targeting the Hippo pathway in the treatment of heart disease.

Keywords

Cardiac regeneration; Hippo pathway; Proliferation; Heart failure; Cell communication; Large animal model

1. Introduction

Heart failure (HF), defined as the inability of the heart to sufficiently pump enough blood to meet the body's needs, is the leading cause of death globally [1]. Currently, an estimated 38 million patients have HF worldwide, and this number continues to rise [2]. Roughly one-half of the patients with HF die within 5 years of diagnosis. HF is a complicated pathological process initiated by cardiac injury, which arises most commonly from ischemic heart disease associated with coronary vascular disease [3]. Although proven treatment options exist that

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Disclosures

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ameliorate the symptoms of HF, therapeutics that can reverse HF or restore damaged heart tissue are critically needed.

In mammals, the heart has a limited regenerative capacity. After cardiac injury, such as myocardial infarction (MI), millions of cardiomyocytes (CMs) die and are not replenished. To date, there are no direct measures to address this CM loss, which is the central underlying mechanism of ischemic HF. Over the past decade, the possibility of repopulating the heart with new CMs after injury has attracted intense interest. Considerable attention has focused on the Hippo pathway, which is a conserved signaling pathway that regulates organ size during development. Studies have revealed that the Hippo pathway is essential for cardiac development, growth, homeostasis, and regeneration [4–12]. In this review, we summarize recent findings regarding pivotal roles of the Hippo pathway in regulating CM renewal and cell communication between different cardiac cells.

2. Cardiomyocyte renewal state in mammals

Heart regeneration has been widely studied for decades. The key question has gradually shifted from whether CMs can proliferate to whether the proliferative capacity of CMs can be optimized for the treatment of various cardiac diseases involving CM loss. During neonatal life, the mammalian heart undergoes hyperplastic expansion and then hypertrophic growth to accommodate the demand for cardiac output [13]. In mice, studies have shown that neonatal CMs undergo DNA synthesis at a rate of 10% during the first 4 days of life; by day 7, this rate is reduced to 1%, indicating near terminal differentiation [14–18]. Studies of CM division have been performed with multi-isotope labeling [19] and a genetic fate-mapping mouse model called mosaic analysis with double markers (MADM) [20]. In both studies, the rate of CM division was analyzed by using lineage tracing and was shown to significantly decrease with age [19,21]. Genetic lineage tracing by using dual recombinases has provided further evidence ruling out the possibility of noncardiomyocyte-to-cardiomyocyte conversion in postnatal mouse hearts [22,23]. A similar phenomenon was observed in humans. In a highly influential study [24], researchers used carbon dating techniques and mathematical modeling to retroactively analyze ^{14}C isotope integration into the CMs of humans who lived at the time of atmospheric nuclear bomb testing during the cold war [24]. They calculated the self- renewal rates of several human cell types, among which CMs were found to be the least renewable cell type [24,25]. At age 20, the renewal rate of CMs is about 1% per year, which decreases with time to ~0.3% by age 70. Thus, an estimated 40% of CMs are generated throughout a full lifespan, whereas the remaining 60% are originally formed during prenatal development [24,25]. Together, these studies have demonstrated that CM proliferation and division are measurable but rare in mammalian hearts.

3. Injury-induced cardiomyocyte proliferation is essential for neonatal heart regeneration

To better understand the role of CM proliferation in heart regeneration, investigators have studied animal models capable of cardiac regeneration, including amphibians and zebrafish [26,27]. Recent reports have shown that spontaneous cardiac regeneration

also occurs in mammalian neonates [18,28]. In either of these contexts, myocardial regeneration is mediated by the compensatory proliferation of preexisting CMs, rather than by stem or progenitor cell populations [29,30]. Studies in zebrafish showed complete cardiac regenerative ability after apical resection, with *Cmlc2a*- or *Gata4*-expressing CMs undergoing proliferation and migrating to the injury site to rebuild the damaged myocardium. Interestingly, the cardiogenesis gene *Gata4* is mainly expressed in epicardial CMs, suggesting injury-induced CM dedifferentiation [29,30]. Independent studies in mice have revealed that the neonatal mouse heart has the capacity to regenerate. Compared with mice that underwent a sham operation, mice with cardiac injury showed a 5-fold increase in CM proliferation within 7 days after birth (i.e., P7) [18,31]. Lineage tracing experiments performed by using inducible *Myh6-MerCreMer* mice crossed with *Rosa26-lacZ* reporter mice showed that the newly generated CMs were primarily derived from preexisting CMs [18,31,32].

Using a transcriptomics approach, Cui et al. [33] compared CMs in a P1 injury versus a P8 injury model and identified an immature CM population (CM4) associated with CM regeneration that was enriched in P1 hearts subject to injury. CM4 is characterized by reduced oxidative metabolism and enhanced cell-cycle activity, along with the increased expression of pro-proliferation and pro-survival factors [33]. These findings suggest that subpopulations of CMs exist in the neonatal heart that maintain an immature gene program and are poised to proliferate in response to injury.

4. Loss of cardiomyocyte proliferation in the adult heart

Because the regenerative window of perinatal murine hearts lasts only a few days, injury in nonregenerative mouse hearts (i.e., age P7 and older) failed to induce CM proliferation, resulting in permanent loss of cardiac muscle [18,31,32]. Similarly, in nonregenerative P7 mice that underwent MI or sham, no difference was observed in the number of phosphorylated histone H3 (pHH3)-positive CMs (pHH3 is a cell cycle marker that is expressed in the G2/M phase, just preceding mitosis) [31]. In another study, CM division detected by using MADM was limited in the hearts of adult mice that underwent MI or a sham operation [20]. Thus, understanding the difference in mechanisms of CM proliferation in neonatal versus adult mouse models is essential for developing therapeutics to induce spontaneous cardiac regeneration in humans.

Although progress has been made, how the heart loses its regenerative ability with age remains poorly understood. Puente et al. [21] reported that the level of reactive oxygen species (ROS) was increased in mice during the first week after birth, which resulted in activation of the DNA damage response in CMs. Reducing ROS level or using compounds to inhibit the DNA damage response extended the window of CM proliferation in the postnatal mouse heart, suggesting that ROS-induced DNA damage plays a role in the transition from the regenerative to the nonregenerative stage in the postnatal heart [21]. Cui et al. [34] recently reported that Nrf1, a stress-responsive transcription factor, regulates proteolysis and redox balance in regenerating CMs. *Nrf1* expression is highest in P1 hearts, specifically in regenerating CMs, and decreases with age. In addition, the adeno-associated adenovirus (AAV)-mediated overexpression of *Nrf1* in the adult heart reduced infarct area

and improved cardiac function after ischemia/reperfusion injury. Transcriptomics and *in vitro* analyses revealed that Nrf1 can activate ROS scavengers and increase proteasomal activity to maintain an environment suitable for CM survival and renewal after injury [34].

As the heart matures during the perinatal period, it undergoes both structural and metabolic changes needed for optimal cardiac function. The sarcomeres and mitochondria of CMs become highly organized and developed, coinciding with the loss of proliferation capability. After birth, mammalian CMs undergo 1 to 2 rounds of cell division before switching to hypertrophic growth [35], which is accompanied by sarcomere myofibrils becoming further organized. This highly complex structure allows CMs to sustain the cycles of contraction and relaxation that compose each heartbeat, but it becomes a hindrance for cell division. Proliferation requires myofibril disassembly and cytoskeletal reorganization, which is challenging in structured and synchronized CMs.

In addition to the rigid sarcomeric cytoskeleton, postnatal metabolism changes also inhibit CM proliferation. Shortly after birth, the metabolic substrate provision changes, which leads to a metabolic switch of CMs from glycolysis to fatty acid oxidation [36,37]. Using an ex vivo three-dimensional cardiac organoid culture system, Mills et al. [38] showed that the switching of metabolic substrate from carbohydrates to fatty acids is a central driver of cardiac maturation, which is accompanied by decreased CM proliferation.

Another potential obstacle for CM proliferation in the adult heart is the change in transcriptional and epigenetic profiles of CMs that occurs beyond the neonatal stage. In a recent study, the authors performed RNA sequencing to examine the transcriptional profiles of CMs and non-CMs from neonatal and adult mouse hearts treated with or without MI [39]. Although injury-responsive genes were identified in regenerative and non-regenerative stages, a regeneration-specific gene profile was not detected. However, in all cardiac cells, the major transcriptional changes observed occurred in the developmental maturation genes from the neonatal stage to adulthood. Further, analysis using the assay for transposase-accessible chromatin sequencing (ATAC-seq) revealed the loss of chromatin accessibility in cell cycle genes. Thus, the change in the epigenetic landscape of CMs during the postnatal maturation phase may be one of the reasons for loss of regenerative capacity. Of note, in this study, cardiac cells were isolated by using enzymatic dissociation and flow cytometry sorting. Therefore, the long isolation process may have altered the physiologic state of the cells, leading to changes in the transcriptional profile that may not fully reflect the transcriptional profile of CMs in vivo [39].

5. Overview of the Hippo signaling pathway

The Hippo pathway is an evolutionarily conserved kinase cascade. In the heart, Hippo pathway activity increases with age, whereas YAP activity decreases [6,9], implying that decreased YAP activity contributes to the loss of regenerative capability in the postnatal heart. In mice, STE20-like kinases 1 and 2 (MST1/2) and their adaptor protein Salvador (SAV1) form an activated complex and phosphorylate the large tumor suppressor homolog 1 and 2 (LATS1/2) and their regulatory protein Mps1-binder-related (MOB) domain kinase activator 1 (MOB1). This kinase cassette further phosphorylates the Hippo pathway's

key effectors Yes-associated protein (YAP)/Transcriptional co-activator with PDZ-binding motif (TAZ), thereby inhibiting the translocation of YAP/TAZ into the nucleus. Non-phosphorylated YAP/TAZ can enter the nucleus, where they function as coactivators with TEA domain (TEAD) transcriptional factors. Together, the YAP/TAZ-TEAD complex induces gene expression programs that favor proliferation and survival [40,41] (Fig. 1).

6. Hippo pathway function in the regulation of cardiomyocyte proliferation

To determine whether modulating Hippo pathway components or increasing YAP activity in CMs promotes heart regeneration, Heallen et al. [5] deleted *Sav1* specifically in embryonic CMs and found evidence of heart expansion during development due to significantly increased CM proliferation. They also deleted other Hippo kinases including *Lats2*, or *Mst1/2*, in embryonic CMs and observed phenotypes similar to those in the *Sav1* mutant [5]. This study showed for the first time that the Hippo-YAP pathway is a critical regulator of CM proliferation and cardiac organ size. Moreover, Xin et al. [11] showed that the deletion of *Yap* in embryonic CMs caused lethality in embryos at E10.5 due to hypoplasia of the myocardium. The number of CMs was decreased by nearly 50% due to significantly decreased proliferation. Consistent with this finding, overexpression of the TEAD1 inhibitor VGLL4 decreased CM proliferation in the neonatal mouse heart. VGLL4 interacts with and degrades TEAD1 in postnatal hearts [42]. Similarly, the deletion of *miR302–367*, which is a negative regulator of the Hippo pathway in CMs, led to decreased CM proliferation and thinning of the ventricular wall. Overexpression of *miR302–367* in CMs promoted proliferation and resulted in cardiomegaly [43]. Deletion of *Yap* in CMs did not affect apoptosis during early heart development, suggesting that YAP regulates cardiac development primarily through its role in proliferation and not through anti-apoptotic functions [9]. Collectively, these studies suggest that the Hippo pathway is essential for restraining CM proliferation during heart development.

The postnatal deletion of *Sav1* or *Lats1/2* leads to YAP/TAZ activation and stimulates CM proliferation in the heart of 3- to 4-month-old mice [6,7]. Similarly, expression of constitutively active YAP (i.e., YAPS127A, with the substitution of serine 127 with alanine to escape phosphorylation) promotes postnatal CM proliferation [9,10]. Monroe et al. [44] generated a mouse strain in which YAP5SA is conditionally expressed in CMs. YAP5SA harbors five serine-to-alanine mutations that interfere with the LATS1/2-mediated phosphorylation of YAP, resulting in increased nuclear YAP activity. The authors found that YAP5SA expression for 6 days increased the number of CMs by nearly 25% due to uncontrolled CM proliferation. All YAP5SA mice died within 8 days after the induction of YAP5SA due to significantly decreased cardiac chamber size and cardiac output. The proliferative CMs were smaller but with similar ploidy compared with the control CMs, with most CMs being mononuclear [44]. Mononuclear CMs may have better renewal capacity than binuclear CMs, and higher ploidy hampers CM renewal in mice and zebrafish [45,46]. Nuclear RNA sequencing in adult CMs expressing YAP5SA revealed a more primitive and proliferative gene profile than adult control CMs. Genes that were upregulated included genes associated with the cell cycle (eg, *Ccna2, Ccnb1, Ccnd1,* and *Myc*) and genes related to cytoskeleton organization, (eg, *Tnni3, Tnnt2, Myh6,* and *Myl2*). ATAC-sequencing showed increased chromatin accessibility at TEAD motifs, indicating that YAP5SA alters

chromatin structure and induces target gene expression to promote CM proliferation [44]. In support of these findings, a recent study demonstrated that YAP directly interacts with the components of the Switch/sucrose non-fermentable (SWI/SNF) chromatin-remodeling complex [47]. These results indicate that activation of YAP can robustly induce adult CMs to re-enter the cell cycle and proliferate in a cell-autonomous manner.

In mice with *Yap* deletion in CMs, no apparent defects were observed during the first few weeks after birth. However, significant thinning of the myocardium wall was seen at 9 weeks of age, and lethality occurred between 11 and 20 weeks [10]. The combined deletion of *Yap* and *Taz* in CMs resulted in lethality by day 1 after birth, suggesting that YAP and TAZ share a redundant role in heart development [10]. Because CMs do not proliferate past P7, the thinning of the myocardium wall after the deletion of *Yap/Taz* may be attributed to both CM proliferation defects and also increased apoptosis. Indeed, increased apoptosis was evident in the hearts of mice with *Yap/Taz* deletion [10]. Consistent with this finding, another group showed that CM apoptosis was increased in *Yap*- deficient mouse hearts, and the knockout of a single allele of *Yap* in CMs resulted in impaired cardiac function after MI [12]. However, these findings are inconsistent with those of von Gise, et al. [9], who showed that the loss of *Yap* does not affect CM apoptosis during development, possibly indicating a stage-dependent role of *Yap* in CMs. Together, these studies suggest that YAP, in addition to promoting proliferation, also may have a role in protecting CMs from apoptosis although further studies are required.

7. The Hippo pathway in the regulation of cardiac regeneration in the context of injury

Analysis of human ischemic and nonischemic HF samples revealed increased Hippo pathway activity when compared with control donor samples [7], suggesting that the Hippo pathway can be targeted as a potential therapy for heart injury and disease. The role of the Hippo pathway in heart regeneration has been tested in several different models. Heallen et al. [6]. examined the capacity for heart regeneration in mice after apical resection and after MI at P8, when the heart has completely lost its regenerative ability [6,18]. In both the resection and MI models, the deletion of *Sav1* in CMs promoted greater heart regeneration with better heart function and less fibrosis than in control hearts [6]. In an adult mouse model of MI with well-established ischemic HF, Leach et al. [7] showed that the knockout of *Sav1* in CMs resulted in the reversal of systolic HF that was not seen in control mice. They also treated mice with AAV9 encoding short hairpin RNA (shRNA) against *Sav1*, which resulted in recovered heart function and CM proliferation. From a mechanistic standpoint, Leach et al. showed that *Park2*, a gene associated with mitochondrial quality control [48], is a target of YAP. CMs with *Sav1* deletion showed higher mitochondrial content than control CMs, supporting the conclusion that maintenance of mitochondrial function may be essential for cardiac regeneration by protecting “at risk” CMs in the border zone. Similar to YAP, Park2 is required for neonatal cardiac regeneration. The knockout of *Park2* in mice at P8 mitigated cardiac regeneration in CMs with *Sav1* deletion [7]. Interestingly, *Park2* deletion diminished heart function in *Sav1* knockout mice, but the fibrosis was still resolved

[7]. Because mitochondria produce energy for CM contraction, the reduced heart function observed after *Park2* deletion may be attributed to insufficient energy supply in CMs.

Using the adult MI mouse model with *Sav1* deletion, Morikawa et al. [8] observed increased CM proliferation after *Sav1* deletion. Moreover, they found extended CM protrusions in the scar region of *Sav1* knockdown hearts compared with control hearts. When they performed YAP chromatin immunoprecipitation sequencing (ChIP-seq) and RNA sequencing of P8 mouse hearts, they observed the upregulation of YAP target genes associated with the cell cycle and cytoskeleton organization in *Sav1* knockout hearts. Among those genes that were upregulated were components of the dystrophin-glycoprotein complex (DGC), such as *Sgcd* and *Sntb1* [49,50]. They also found that the DGC is essential for the regulation of CM protrusion in neonatal heart regeneration via a cell- autonomous mechanism [8].

Morikawa et al. [51] further uncovered the connection between the Hippo pathway and the DGC in cardiac regeneration by showing that the DGC directly interacts with YAP and prevents the nuclear localization of YAP. DGC deficiency results in muscular dystrophy, which is usually associated with dilated cardiomyopathy. In *mdx* mice with the mutation of dystrophin, the combined knockout of *Sav1* resulted in higher YAP activity than in *Sav1* single knockout mice. Double knockout mice were also resistant to dystrophin deficiency–induced fibrosis due to increased YAP activity [51]. In line with this study, Bassat et al. [4] discovered that Agrin, a component of the extracellular matrix (ECM), interacts with the DGC and disassociates the YAP/DGC complex, thereby releasing YAP to the nucleus and promoting CM proliferation. Since Agrin is a component of the extracellular matrix and is expressed by endothelial cells, the study by Bassat et al. provides important new insight into the role of the extracellular microenvironment in CM proliferation.

In neonatal mouse hearts still within the window of regenerative capability, the deletion of *Yap* impaired regeneration after MI at P2 [10]. In injured adult mouse hearts, the heterozygous deletion of one allele of *Yap* in CMs resulted in increased scar size and decreased heart function compared with controls due to significantly increased CM apoptosis [12]. Conversely, the overexpression of YAP with enhanced activity (YAPS112A, a homolog of human YAPS127A) specifically in CMs improved heart regeneration after MI induced at P7 or P28 in nonregenerative mouse hearts [10]. Moreover, using a doxycycline-inducible *YapS127A* mouse strain, Lin et al. [52] showed that YAP activation was sufficient to promote heart regeneration in adult mouse hearts.

During the first week after birth, cardiac levels of ROS are increased. ROS can induce the DNA damage response in CMs and suppress cardiac renewal [21]. Shao et al. [53] reported that ROS levels are increased in the hearts of mice with the cardiac-specific deletion of *Yap*. They also found that YAP directly interacts with forkhead box protein O1 (FOXO1) to induce antioxidant gene expression. Similarly, Tao et al. [54] showed that paired like homeodomain 2 (PITX2) interacts with YAP and induces the expression of antioxidant genes such as *Ldha*, *Ndufb3*, and *Oxnad1*. Deletion of *Pitx2* in CMs impaired neonatal heart function recovery and increased scar formation after apical resection or MI, suggesting an essential role for the anti-oxidative response induced by the PITX2-YAP interaction in heart regeneration [53,54]. In addition as noted above, Leach et al. [7] observed increased

mitochondrial content in *Sav1* knockout CMs. Thus, the mitochondria-derived ROS may be resolved by YAP-induced antioxidant genes. Together, these results suggest that the Hippo pathway regulates both CM proliferation and antioxidation during cardiac regeneration.

8. The non–cell-autonomous role of the Hippo pathway in regulating cell-to-cell communication

In addition to its cell-autonomous role in regulating CM proliferation, the Hippo pathway has been shown to regulate cell-to-cell communication from one cell type to another (Fig. 2). During cardiac regeneration, the re-establishment of new vasculature and fibrosis resolution are critical to repairing damaged heart tissue. Leach et al. [7] showed that the CM-specific knockout of *Sav1* not only promoted CM proliferation, but also increased border zone vascularity and reduced fibrosis. These results suggest that CMs may signal to endothelial cells and fibroblasts via a non–cell-autonomous manner during cardiac renewal.

To identify the YAP targets associated with extracellular signaling transduction, Liu et al. [55] examined YAP targets in mouse hearts with CM-specific overexpression of YAP5SA. By combining nuclear RNA sequencing, ATAC-seq, and ChIP-seq datasets, Liu et al. [55] showed that Wnt signaling pathway genes are direct downstream targets of YAP. The expression of *Wntless (Wls)*, which is essential for the trafficking and secretion of Wnt ligands, and several Wnt ligands was significantly upregulated, suggesting a direct connection between the Hippo and Wnt signaling pathways. Using both CRISPR/Cas9 and traditional Cre/LoxP mouse models, we found that Wls is required for neonatal heart regeneration and scar resolution. Using single-cell RNA sequencing, we observed higher expression of noncanonical Wnt ligand genes than canonical Wnt ligand genes in CMs. We also found that Wnt receptors are highly enriched in fibroblast cells, suggesting that noncanonical Wnt signaling from CMs to fibroblasts is essential for cardiac homeostasis and regeneration. Further analysis indicated that impaired heart regeneration was due to excessive cardiac fibrosis after *Wls* deletion and that noncanonical Wnt signaling from CMs to fibroblasts is essential for inhibiting fibroblast activation in neonatal heart regeneration [55]. This study not only uncovered the role of the Hippo-Wnt gene regulatory network in the cell communication between CMs and fibroblasts, but it revealed noncanonical Wnt signaling from CMs acting as a non–cell- autonomous regulator to suppress cardiac fibroblast activation. In addition to CMs, other cell types also secrete Wnt proteins, such as fibroblasts and epicardial cells. Determining how Hippo regulates Wnt signaling from different sources and how these signals affect heart regeneration and scar resolution requires further study. Additionally, examining the expression and role of each of the 19 Wnt ligands in the heart remains to be done.

The Hippo signaling pathway also plays a role in communication between the epicardium and other cell types. The epicardium covers the outer layer of the heart and functions as a source of multipotent progenitor cells and paracrine factors essential for cardiac development and repair [56–61]. Xiao et al. [62] showed that the Hippo pathway is essential for epicardial cells to differentiate into cardiac fibroblasts. Epicardial *Lats1/2* knockout resulted in the expansion of an intermediate cell type between epicardial cells and cardiac

fibroblasts. This was accompanied by altered ECM composition and suppressed coronary vessel patterning that occurred in a non–cell-autonomous manner. Consistent with this finding, Singh et al. [63] showed that mice with the epicardial-specific double knockout of *Yap/Taz* died around E11.5 to E12.5 from cardiovascular hypoplasia due to defects in epicardial cell proliferation, epithelial-to-mesenchymal transition, and fate determination. Using the same *WT1-Cre* mice, Ramjee et al. [64] deleted *Yap/ Taz* specifically in epicardial cells and observed profound pericardial inflammation and myocardial fibrosis after MI due to decreased T-reg cell recruitment. In addition, they identified interferon gamma (IFN-γ) as a direct target of *Yap/Taz* that functions as a T-reg inducer [64–68]. Together, these studies have demonstrated the essential role of the Hippo pathway in the communication of the epicardium with other cells in the heart through non–cell-autonomous mechanisms, including immune cells, endothelial cells, and fibroblasts (Fig. 2).

Cardiac fibroblasts respond to injury by transitioning through different cell states, including resting cardiac fibroblasts, activated fibroblasts, and myofibroblasts. Using *Tcf21-Cre* mice, Xiao et al. [69] studied *Lats1/2* knockout cardiac fibroblasts and observed spontaneous fibroblast proliferation, activation, and transdifferentiation into myofibroblasts. By performing ATAC-seq and CUT&RUN, they identified direct targets of the Hippo pathway associated with fibroblast activation and the inflammatory response. Single-cell RNA sequencing analysis indicated *Lats1/2* knockout myofibroblasts recruited myeloid cells through the Csf1-Csf1r axis. Consistent with this, Francisco et al. [70] overexpressed YAP in cardiac fibroblasts by using AAV and observed an increase in fibrosis and the inflammatory response in the heart. In determining the mechanism, they found that YAP occupied the *Ccl2* gene and promoted *Ccl2* expression. Ccl2 is critical for monocyte and macrophage recruitment, which is in turn essential for cardiac inflammation, remodeling, and regeneration [71–74]. Moreover, Mia et al. [75] reported that YAP/TAZ directly regulate the promoter activity of the profibrotic cytokine interleukin-33 (IL-33) in cardiac fibroblasts. Blocking the IL-33 receptor ST2 by using a neutralizing antibody abrogated the YAP-induced profibrotic response in cardiac fibroblasts. In another study, Del Re et al. [76] reported that the Ras-associated domain family 1 isoform A (RASSF1A), which is an endogenous activator of MST1, inhibits nuclear factor kappa B (NF-κB) and tumor necrosis factor alpha (TNF-α) in cardiac fibroblasts [77,78]. Treating *Rassf1a* knockout mice with TNF-α antibody decreased transverse aortic constriction–induced fibrosis [76]. Together, these studies show that the Hippo pathway regulates the cell state transition of fibroblasts and the immune response in the context of both homeostasis and injury.

Reports have shown that the Hippo pathway plays a pivotal role in endothelial cells during heart development. Using an endothelial *Tie2-Cre* mouse, Zhang et al. [79] knocked out YAP in the endothelial and endocardial cells of the developing heart. This conditional mouse strain was embryonic lethal due to vascular abnormalities and impaired heart function. The conditional deletion of YAP not only decreased endothelial cell proliferation, but also impaired the endothelial-to-mesenchymal transition in a cell-autonomous manner [79]. In addition, Artap et al. [80] specifically knocked out YAP/TAZ in endocardial cells using *Nfatc1*$^{IRES\text{-}Cre}$ mice. Consistent with previous findings showing that the coronary endothelium arises from *Nfatc1*-expressing endocardial cells [81], Artap et al. found that endocardial-specific YAP/TAZ deletion caused early postnatal lethality due to cardiac

hypoplasia. They further showed that YAP directly induces the expression of NRG1, which is a secreted factor that plays an essential role in myocardium development [82–84] (Fig. 2).

9. The translational potential of Hippo pathway inhibition in a pig model of cardiac renewal

To date, the animal models most commonly used for studying CM renewal have been mice and zebrafish. Although these animal models have provided valuable insights, they have limitations for translational studies because their cardiovascular anatomy and physiologic features are distinctly different from those of the human heart [85,86]. Pig models have become highly valuable in translational cardiovascular research. The physiology, heart size, immune system, and anatomy of the pig heart closely resemble those of the human heart. For example, pig and human hearts have similar contractile indices, determined by using cardiac catheterization measurements [87,88]. Pig and human CMs also share many characteristics in excitation-contraction coupling. Furthermore, similar to human CMs, pig CMs predominantly express β-myosin heavy chain, and stiff N2B and N2BA titin isoforms are both expressed in pig myocytes [87]. Pigs also share similar regional cardiac hemodynamic features with humans [89,90], and both pig and human hearts have reduced contractility after MI that is caused by alterations in Ca^{2+} handling [86]. Increased Ca^{2+} sensitivity is also a common feature in pig and human hearts after MI [88].

Velayutham et al. [91] investigated the characteristics of CMs, including cell cycle arrest, nucleation, and hypertrophy in postnatal pigs. Surprisingly, unlike mouse CMs, which lost cell cycle activity within several days after birth, pig CMs showed mitotic activity for up to 2 months of age. This mitotic activity may have resulted from nuclear division (karyokinesis) but not cell division (cytokinesis) and was accompanied by CM hypertrophic growth and multinucleation. At 6 months of age, most pig CMs are multinucleated, with a maximum of 16 nuclei per CM.

Although cell cycle activity is sustained in CMs of 2-month-old pigs, several independent studies have shown that cardiac regenerative ability lasts only up to 2 days after birth [28,92,93]. To examine the translational potential of targeting the Hippo pathway to promote the regeneration of pig hearts, Liu et al. [94] used an AAV9-mediated gene therapy strategy to knockdown the Hippo pathway component *Sav1* in CMs. Three-month-old pigs were treated with an angioplasty balloon to induce ischemic/reperfusion injury. Two weeks after MI, NOGA electromechanical mapping was used in combination with a Myostar catheter to detect the infarcted border zone region of the myocardium and deliver AAV9-encoding shRNAs against *Sav1* into CMs [94,95]. This noninvasive method resulted in high-efficiency AAV9 viral infection and the nuclear localization of YAP. Compared with the controls, pig hearts injected with *AAV9-Sav-shRNA* recovered nearly 10% ejection fraction during the three-month follow-up period. In pig hearts that received a higher dosage of the *AAV9-Sav-shRNA* virus, the ejection fraction was increased by 14%. In the clinical setting, this degree of functional recovery could significantly improve quality of life for patients with MI. We also analyzed CM proliferation by using multiple methods and observed significantly increased cell cycle activity in CMs injected with *AAV9-Sav-shRNA*. Furthermore, CM

dedifferentiation and division was detected in pig hearts injected with *AAV9-Sav-shRNA* but not in control pig hearts. The effects of *AAV9-Sav-shRNA* appeared to be CM specific, with no abnormalities or tumor formation detected in other organs such as liver, lung, or kidney during the three-month follow-up period [94]. Notably, the divided CMs had fully matured sarcomere structure and normal gap junction localization, suggesting that the newly formed CMs had coupled and integrated into the myocardium [94].

In a study by Zhao et al. [96], human induced pluripotent stem cell (iPSC)-derived CMs were transplanted into the infarcted hearts of two-month-old pigs. They found that iPSC-derived CMs overexpressing *CCND2* could continue to proliferate after being engrafted to the infarcted myocardium. Furthermore, the iPSC-derived CMs secreted exosomes with miRNAs and promoted cardiac regeneration by stimulating the proliferation of pig CMs and endothelial cells while reducing CM apoptosis. In further mechanistic studies, they found that the expression of *miR-373–302b* is upregulated in exosomes from iPSC- derived CMs overexpressing *CCND2*. miR-373–302b inhibits the Hippo pathway and activates YAP activity, which in turn promotes cell proliferation and survival [96].

In another study in pigs, Gabisonia et al. [97] injected AAV6 expressing *microRNA (miR)-199a* into infarcted pig hearts. The known targets of miR-199a include two Hippo pathway inhibitors, TAO kinase 1 [98,99] and the E3 ubiquitin-ligase β-transducing repeat containing protein [100]. Consistent with studies in mice [101–104], they observed CM proliferation, cardiac repair, and reduced scar size in pig hearts one month after *AAV6-miR-199a* injection. However, the subsequent persistent and uncontrolled expression of *miR-199a* caused sudden arrhythmic death within two months of viral injection [97]. Similarly, studies in mice have also shown that the constitutive overexpression of YAP or miRNAs in the heart can be deleterious [43,44]. The reason for this may be that miRNAs have hundreds of conserved target genes; thus, negative off-target effects may occur due to miRNA overexpression. Unlike the effect of miRNAs, the knockdown of Hippo pathway components may activate a negative regulation mechanism to constrain the persistent activity of YAP. Indeed, YAP-LATS2 have been shown to form a negative feedback loop [105]. Collectively, these studies support the conclusion that targeting the Hippo pathway to treat heart disease can be done in an effective and safe manner in the translational context of a large animal model.

10. Future directions

Several studies have shown that increasing YAP activity in CMs has the potential to promote cardiac regeneration. However, targeting the Hippo-YAP pathway in a general manner to treat heart disease is impractical because YAP is an oncogene in some tissues [106]. Moreover, the knockout of Hippo pathway components in cardiac fibroblasts causes fibrosis and inflammation, which would impair cardiac regeneration [69]. Thus, activating YAP in a CM-specific manner may be a better strategy for developing treatments for patients with HF. Unlike other cell types, CMs are highly structured cells packed with dense cytoskeleton networks [107,108]. Studies have shown that several cytoskeletal components including α-catenin, and the DGC physically interact with YAP and prevent its nuclear localization in CMs and other cell types [4,7,109–112]. Because the Hippo pathway is involved in

sensing mechanical stress [40,113–116], investigating the connection between the CM cytoskeleton and Hippo pathway regulation would be of great interest. Moreover, CMs are the most energy-demanding cells. In the setting of MI, metabolism quickly shifts from mitochondrial oxidative phosphorylation to glycolysis because of CM oxygen deficiency [117–119]. Understanding whether and how the Hippo pathway affects or is affected by metabolism in the context of the post-MI heart requires further study.

Another strategy to circumvent the systemic activation of YAP would be to identify YAP target genes during CM proliferation and directly induce the expression of those YAP target genes to stimulate CM proliferation. Although several studies have shown many YAP target genes to be associated with the cell cycle [8], how YAP regulates the gene regulatory network to induce cell cycle entry and cell division remains poorly understood. In addition, in contrast to fetal and neonatal CMs, mature adult CMs must overcome the structural block to cell division. Dissecting the cell cycle process from entry to division in adult CMs would be essential to developing strategies to stimulate CM proliferation in human patients.

Recent studies have investigated the role of the immune system in CM proliferation, comparing the differences in the cardiac immune response after injury between renewable and nonrenewable models [73,120–122]. These studies highlight subpopulations of macrophages, which are rare in the adult heart, that may facilitate or support CM renewal. Results from other studies have revealed essential roles for YAP in CMs, epicardial cells, and fibroblasts that lead to differential immune cell recruitment and proliferation [44,64,69]. In turn, these myeloid cells signal to other cell types in the microenvironment. Further investigation into the connection between YAP and macrophages and other inflammatory cells in the cardiac microenvironment is warranted.

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Abbreviations:

AAV9	adeno-associated virus 9
ChIP	chromatin immunoprecipitation sequencing
CM	cardiomyocyte
DGC	dystrophin-glycoprotein complex
ECM	extracellular matrix

FOXO1	forkhead box protein O1
HF	heart failure
HFrEF	heart failure with reduced ejection fraction
IFN-γ	interferon gamma
IL-33	interleukin-33
iPSC	induced pluripotent stem cell
MADM	mosaic analysis with double markers
MI	myocardial infarction
MOB	Mps1-binder-related
NF-κB	nuclear factor kappa B
PITX2	paired like homeodomain 2
ROS	reactive oxygen species
shRNA	short hairpin RNA
SWI/SNF	Switch/sucrose non-fermentable
AZ	Transcriptional co-activator with PDZ-binding motif
TEAD	TEA domain
TNF-α	tumor necrosis factor alpha
YAP	yes-associated protein

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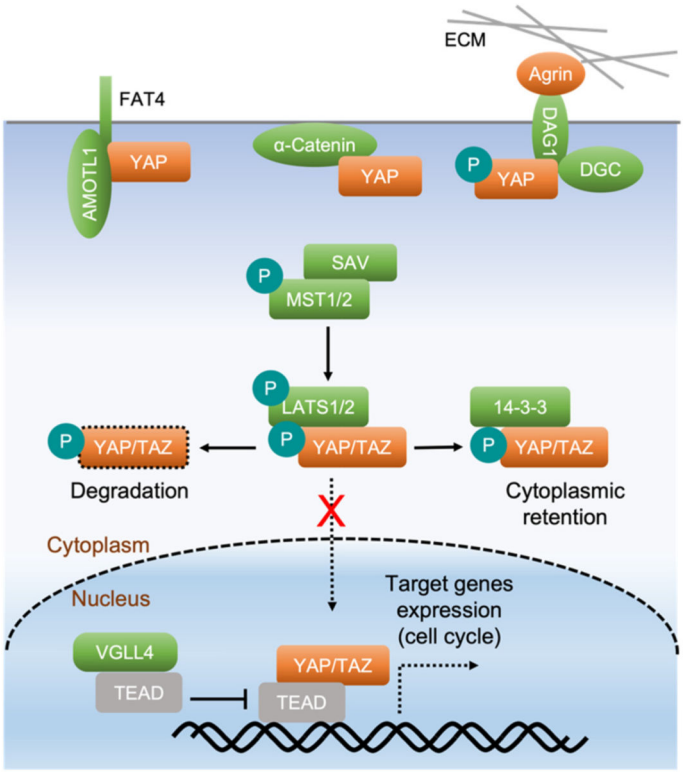

Fig. 1.

The Hippo pathway regulates cardiomyocyte proliferation. The Hippo pathway is composed of several core components: MST1/2, SAV, and LATS1/2. Kinases MST1/2 interact with their adaptor SAV and phosphorylate and activate kinases LATS1/2 that further interact with and phosphorylate YAP and its analog TAZ. Phosphorylated YAP/TAZ are bound and sequestered by 14–3–3 in the cytoplasm or undergo ubiquitination and degradation. When the Hippo pathway is inactivated, unphosphorylated YAP/TAZ translocate into the nucleus, where they interact with their binding partners, such as TEADs, to regulate the expression of downstream target genes that promote cardiomyocyte proliferation. YAP directly interacts with α-catenin, the FAT4 and AMOTL1 complex, and the dystrophin glycoprotein complex (DGC). This causes YAP cytoplasmic retention at the cell membrane, which regulates YAP activity through a Hippo-independent mechanism. The extracellular matrix (ECM) component Agrin physically interacts with the DGC (DAG1 is a protein of the DGC) and disrupts the YAP-DGC interaction, leading to YAP activation.

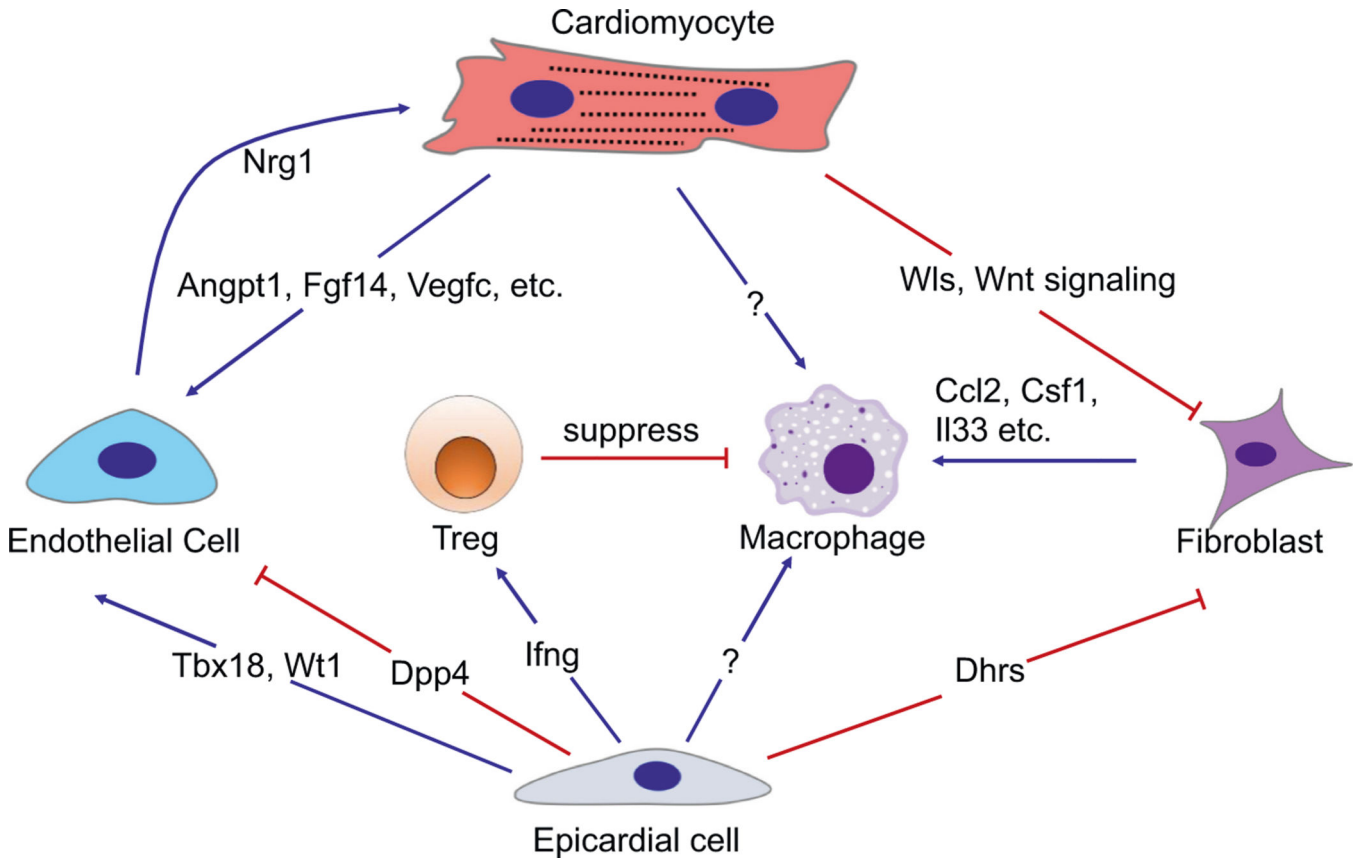

Fig. 2.

The Hippo pathway regulates cell-to-cell communication in the heart. Cell-to-cell communication is essential for cardiac homeostasis and regeneration. In cardiomyocytes, Hippo/YAP induce *Wls* and Wnt signaling to suppress fibroblast activation in neonatal heart regeneration. *Sav1* knockout activates the expression of genes such as *Angpt1*, *Fgf14*, and *Vegfc* in cardiomyocytes [7], which in turn promotes vascularity in the infarcted area. Epicardial cells function as a source of multipotent progenitor cells and paracrine factors essential for cardiac development and repair. YAP regulates epicardial cell proliferation, the epithelial-to-mesenchymal transition, and differentiation into endothelial cells by modulating *Wt1* and *Tbx18* expression. The Hippo pathway also regulates *Dpp4* expression in epicardial cells. Dpp4 proteolyzes both extracellular matrix and matrix- embedded growth factors to modulate endothelial cell migration [123]. *Dhrs3* was upregulated *in Lats1/2* knockout epicardial cells, which may contribute to impaired fibroblast differentiation by reducing retinoic acid formation and signaling [124]. In epicardial cells, YAP/TAZ induce IFN-γ and recruit T-reg cells to suppress the inflammatory response and fibrosis after myocardial infarction. In fibroblasts, Hippo/YAP induce the expression of cytokines including *Ccl2*, *Csf1*, and *IL-33* to recruit proinflammatory macrophages. In endothelial cells, YAP induces *Nrg1* expression, which is essential for myocardial development [80].