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Review

Cardiac regeneration: Unraveling the complex network of intercellular crosstalk

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ABSTRACT

The heart is composed of multiple cell types, including cardiomyocytes, endothelial/endocardial cells, fibroblasts, resident immune cells and epicardium and crosstalk between these cell types is crucial for proper cardiac function and homeostasis. In response to cardiac injury or disease, cell-cell interactions and intercellular crosstalk contribute to remodeling to compensate reduced heart function. In some vertebrates, the heart can regenerate following cardiac injury. While cardiomyocytes play a crucial role in this process, additional cell types are necessary to create a pro-regenerative microenvironment in the injured heart. Here, we review recent literature regarding the importance of cellular crosstalk in promoting cardiac regeneration and provide insight into emerging technologies to investigate cell-cell interactions *in vivo*. Lastly, we explore recent studies highlighting the importance of inter-organ communication in response to injury and promotion of cardiac regeneration. Importantly, understanding how intercellular and inter-organ crosstalk promote cardiac regeneration is essential for the development of therapeutic strategies to stimulate regeneration in the human heart.

1. Introduction

Multicellular organisms rely on cell-cell communication for their development, maturation, survival and homeostasis. The ability of cells to send and receive signals is essential and can occur through cell-cell contact (juxtacrine), ligand-receptor-mediated signaling (autocrine, paracrine), or through secretion of extracellular matrix (ECM) proteins or extracellular vesicles (EVs). With the advent of single-cell and spatial transcriptomic approaches combined with refined bioinformatic algorithms to infer cell-cell communication (recently reviewed here [1]) and 3D organoid models with defined molecular and cellular contributions, we have gained extraordinary insight into potential mechanisms of cell-cell communication in various tissue and organ types. Furthermore, with the advance of imaging techniques and novel synthetic systems to track cell-cell interactions (CCIs, recently reviewed here [2]), it is possible to visualize these interactions within an organism or tissue type with unprecedented detail.

The cardiovascular system is one of the first organ systems to form in the developing embryo and proper development, maturation, and homeostasis of the cardiovascular system is essential for organismal health and survival. The heart is composed of multiple cell types, including cardiomyocytes, endothelial/endocardial cells, fibroblasts, epicardial cells, perivascular cells, lymphatic cells, and tissue-resident immune cells. Adult mammalian hearts have limited capacity for regeneration, as cardiomyocytes (CMs) permanently exit the cell cycle shortly after birth [3,4], and CM loss following injury ultimately leads to heart failure. Zebrafish exhibit the remarkable ability to regenerate the ventricle following injury, as evidenced by recovery of cardiomyocyte cell number and ventricular morphology 30–60 days post injury [5–10]. Similarly, neonatal mouse hearts generate a robust regenerative response following injury, which is correlated with global activation of CM proliferation [11,12]. Endogenous cardiac regeneration requires that CMs

Abbreviations: CCI, cell-cell interaction; cECs, coronary endothelial cells; CM, cardiomyocyte; EC, endocardial cell; ECM, extracellular matrix; EV, extracellular vesicle; FACS, fluorescence activated cell sorting; Fb, fibroblast; HUVEC, human umbilical vein endothelial cell; LAD, left anterior descending; MI, myocardial infarction; NRVM, neonatal rat ventricular myocyte; scRNA-seq, single-cell RNA-sequencing; T_{reg}, regulatory T cell.

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undergo dedifferentiation, characterized by embryonic gene reactivation and sarcomere disassembly, and proliferation to restore lost CMs [13–16]. Further, CM protrusion and invasion into the wound is essential for resolution of injured tissue during regeneration [17–20]. More recent studies have shown that CM redifferentiation to a mature CM state is an essential component of the regeneration process and is necessary for proper calcium handling, cardiac dyad formation, and functional output following cardiac injury [21,22]. Despite the requirement of CMs in cardiac regeneration, the importance of additional cardiac cell types in orchestrating the regenerative response to injury has become increasingly clear. In this review, we highlight recent advances in our understanding of CCIs, cell-ECM interactions, and

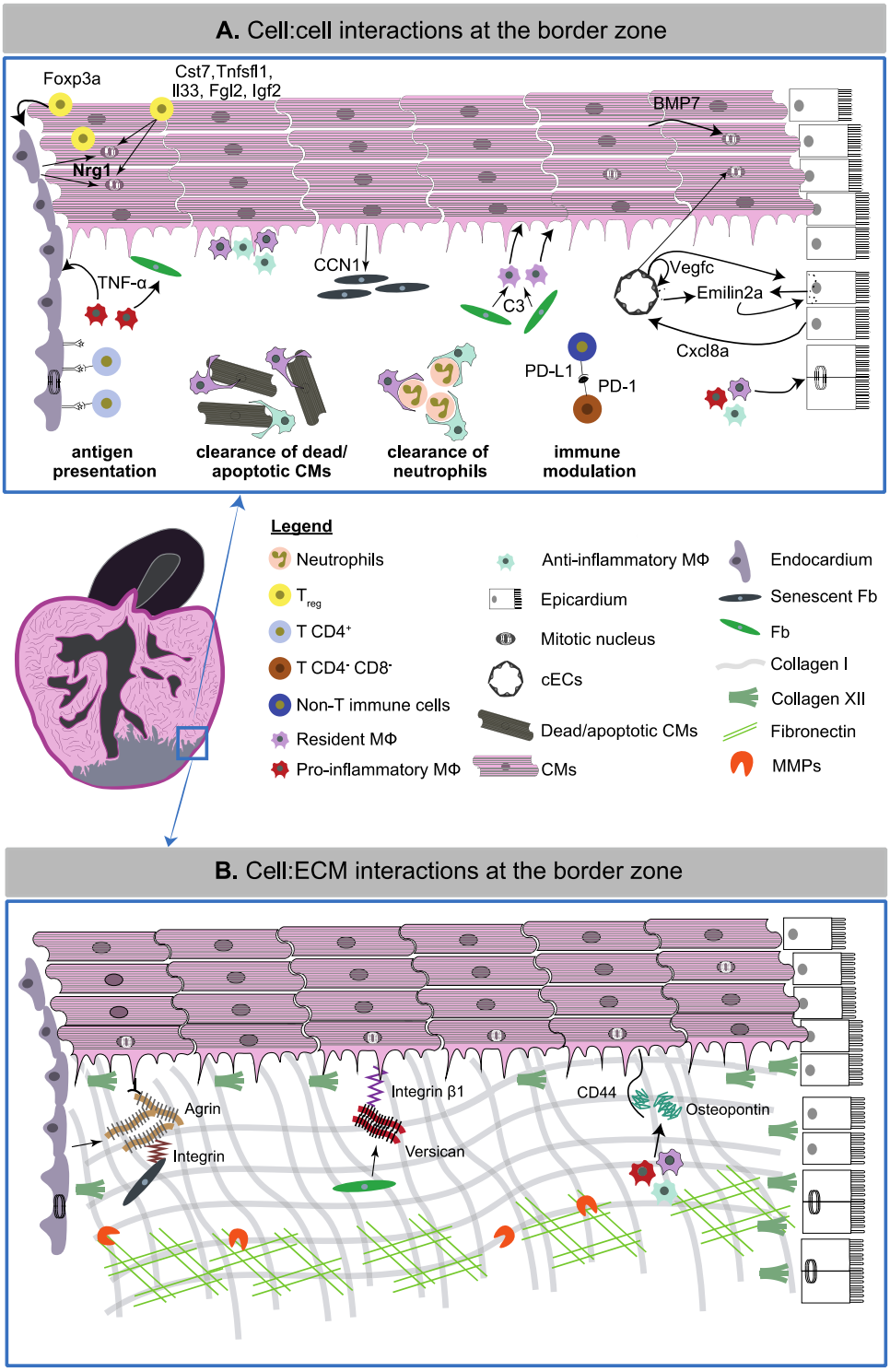

Fig. 1. Establishment of a regenerative microenvironment through cell-cell and cell-ECM interactions.**(A)** Schematic illustrating key cell-cell interactions at the wound border zone (blue box) driving heart regeneration following myocardial injury. Cell types are described in the legend and arrows depict the direction of regulation by implicated ligands. **(B)** Schematic illustrating key ECM components at the wound border zone (blue box) supporting heart regeneration. Cell types are described in the legend and arrows depict the direction of regulation by implicated ECM proteins. T_{reg}, regulatory T-cell; Mϕ, macrophage; cECs, coronary endothelial cells; CMs, cardiomyocytes; Fb, fibroblast; MMPs, matrix metalloproteases.

intercellular crosstalk, and the mechanisms by which these interactions regulate cardiac regeneration. Further, we examine technical strategies to investigate CCIs and discuss their utilization in the heart.

2. Cellular crosstalk in cardiac regeneration

While CM regeneration is one of the central pillars of restoration of cardiac function following injury, additional cardiac cell types have been shown to provide signals to instruct CM regeneration and form a microenvironment to promote cardiac regeneration. Innate immune cells are one of the first cell types to respond to injury, and have been shown to play an important role in stimulating revascularization, removing cellular debris due to injury, and activating epicardial and endocardial cells. Epicardial and endocardial cells provide a cellular source for growth factors and signaling to promote CM proliferation, while fibroblasts derived from both the epicardium and endocardium deposit ECM to stabilize injured tissue during the regeneration process and promote cardiac regeneration. ECM remodeling and degradation during later stages of regeneration, primarily mediated by macrophages, provide a permissive environment to facilitate replenishment of injured tissue with newly generated CMs in order to restore cardiac function following injury. Cardiac regeneration is a highly orchestrated process requiring crosstalk between several cardiac cell types to ultimately restore the function of the heart. We discuss these CCIs and mechanisms by which intercellular crosstalk promotes cardiac regeneration in detail below (Fig. 1A and Table 1).

2.1. The immune response

The early phase in response to heart injury is characterized by activation of the innate immune response. Release of damage-associated molecular patterns from dying cardiac cells stimulates the early recruitment of neutrophils, which function to clear necrotic tissue and cellular debris. Apoptotic neutrophils in the injured heart are phagocytosed by macrophages, and this phagocytosis has been shown to polarize macrophages towards a reparative phenotype [23]. Macrophages are essential regulators of the cardiac regeneration process in zebrafish, salamander, and neonatal mouse hearts [24–26], and function to secrete molecules that promote CM proliferation [27,28], and directly regulate collagen dynamics in the injured area [29]. We have recently shown that during zebrafish heart regeneration, macrophages interact closely with protruding CMs at the border zone, and their presence is essential for regulating the ECM in the injured area and CM protrusion length [19]. We hypothesize that this CCI is crucial for replacement of injured tissue with newly generated CMs.

More recent studies have shown that adaptive immune cells, namely T cells, also play an important role in the response to cardiac injury and promotion of regeneration. Programmed Death-1 (PD-1) signaling is induced following heart injury [30]. In the regenerative neonatal mouse heart at postnatal day 2 (P2), PD-L1 ligand is expressed in non-T immune cells in response to MI, while PD-1 receptor expression is restricted to $CD4^-$ $CD8^-$ double negative T cells in injured hearts. PD-1/PD-L1 signaling between non-T and double negative T cells is essential for neonatal heart regeneration, as abrogation of this signaling through treatment of mice with a PD-L1 antibody or deletion of the ligand-binding and transmembrane domains in *Pdcd1* (encoding PD-1) leads to defects in CM proliferation and resolution of fibrosis following left anterior descending (LAD) artery ligation [31]. Mechanistically, blocking the PD-L1/PD-1 signaling axis leads to activation of double negative T cells and exacerbation of proinflammatory signaling in the neonatal heart, indicating that CCIs and crosstalk between immune cells dampen the immune response to create a regeneration-permissive environment. In the regenerating zebrafish heart, a recent study has also shown that *cd74*, known for its essential role in antigen presentation, is required for $CD4^+$ T cell infiltration, endocardial cell (EC) proliferation, and CM dedifferentiation and proliferation [32]. Notably, *Cd74* is also found activated in EC during neonatal mouse heart regeneration following injury [32].

Regulatory T cells (T_{reg} cells, $CD4^+$ $Foxp3^+$ T cells) have also been shown to promote CM proliferation in a paracrine manner in the zebrafish heart, mouse heart, and human embryonic stem cell-derived CMs [33–35]. In the regenerating zebrafish heart, depletion of T_{reg} cells leads to formation of a thinner myocardial wall with fibrin deposition and compromised scar resolution, and attenuated CM proliferation via Nrg1-ErbB signaling [33]. T_{reg} cells are also essential for neonatal mouse heart regeneration [35], and single-cell RNA-sequencing (scRNA-seq) data revealed the significant upregulation of several ligand-receptor pairs - Ccl24-CCR, Gas6-AXL, Grn-EphA2 and Areg-EGFR, in T_{reg} cells and CMs, that potentially mediate crosstalk within the injured heart [35]. In the nonregenerative adult murine heart, T_{reg} cells contribute to MI healing by resolving inflammation, improving collagen deposition, and promoting CM proliferation via secreted factors such as Cst7, Tnfsf11, Il33, Fgl2, Matn2, and Igf2 [34]. Altogether, the studies described here provide important insight into the immune response, and how cellular crosstalk between immune cells and other cell types in the heart can promote regeneration and repair. A more detailed overview of the immune response in cardiac development and regeneration can be found in the review by Byatt *et al.* in this special issue [36] and in a previously published review [37].

2.2. Revascularization

In response to heart injury, angiogenesis in the ischemic/necrotic region is vital for re-oxygenating the tissue and supplying nutrients to support heart regeneration. In the regenerating zebrafish heart, rapid revascularization of the injured tissue, likely through the migration of existing coronary vessels, is critical for heart regeneration [38]. Revascularization in zebrafish involves a coordinated multi-cellular response and can be divided into two key steps: superficial sprouting in the regenerating epicardium and intra-ventricular sprouting [39]. The first step is potentially regulated by Apelin signaling and the guidance of Cxcr4b+ coronary endothelial cells (cECs) by Cxcl12b from the epicardium. Subsequently, *vegfaa* expression in the endocardium drives intra-ventricular sprouting of cECs [39]. The authors also show that CMs are in close proximity to coronary vessels during cardiac development and regeneration and hypothesize that these vessels promote repopulation of the injured tissue with new CMs. In the regenerating neonatal mouse heart, collateral arteries provide alternative blood flow through the reassembly of arterial vessels by the fusion of non-ischemic and ischemic vessels. This process is driven by the upregulation of *Cxcl12* in capillary cECs and *Cxcr4* in arterial cECs [40], and is reviewed in detail by Ghosh *et al.* in this issue [41].

The importance of revascularization in response to injury has been further demonstrated by the stimulation of regeneration by cECs in a paracrine manner. For example, Vegfc signaling drives a complex network of autocrine and paracrine interactions essential for establishing a regenerative environment in the zebrafish heart [42]. *vegfc* upregulation in cECs triggers *emilin2a* expression in cECs and epicardium-derived cells, and blocking Vegfc and *emilin2a* leads to defects in CM dedifferentiation and proliferation [42] (Fig. 1A). Further evidence of endothelial cell:CM interaction in promoting regenerative hallmarks was shown in co-culture of human umbilical vein endothelial cells (HUVEC) and neonatal rat CMs. Overexpression of endothelial-enriched transcription factors that are predicted to regulate chromatin dynamics in regenerating hearts in HUVECs stimulates proliferation of co-cultured CMs *in vitro* [43].

2.3. Endocardium and epicardium

The endocardium is an endothelial cell layer lining the chambers of the heart and has been shown to play an essential role in regeneration through the upregulation of Notch signaling [44–46]. Notch proteins are

Table 1
Summary of cell-cell interactions in the heart. cECs, coronary endothelial cells; CMs, cardiomyocytes; EC, endocardial cell; ECM, extracellular matrix; Fb, fibroblast; KO, knockout; MI, myocardial infarction; Ref, reference; TF, transcription factor; T_{reg}, regulatory T cell.

	Cell-cell interaction	Signaling pathway involved	Impact on cardiac regeneration/repair	Technology used to describe the interaction	Model	Ref
Immune cells						
	apoptotic neutrophils – macrophages		Phagocytosis of neutrophils leads to macrophage polarization towards a reparative phenotype during cardiac repair	Neutrophil depletion by monoclonal Ly6G antibody injection	Adult mice	**23**
	macrophages – CMs	Oncostatin M – gp130	Promotes CM proliferation in the nonregenerative adult murine heart	RNA-seq and conditional KO	Adult mice	**27**
	macrophages – CMs		Promotes protrusion of border zone CMs into the injured tissue in regenerating hearts	Genetic and pharmacological depletion of macrophages	Zebrafish	**19**
	double negative T cells – non-T immune cells	PD−1/PD-L1	Dampens the immune response to establish a regeneration-permissive environment in regenerating hearts	scRNA-seq, genetic deletion, and neutralizing antibody	Neonatal mice	**31**
	EC – CD4 + T cells	Cd74 pathway, MHC II – T cell receptor	Antigen presentation required for T cell infiltration, EC proliferation, and CM dedifferentiation in the regenerating heart	scRNA-seq and genetic deletion	Zebrafish	**32**
	Treg – CMs	Nrg1 – ErbB	Regulates CM proliferation in the regenerating heart	Cell ablation and rescue experiments	Zebrafish	**33**
	Treg – CMs	Cst7, Tnfsf11, Il33, Fgl2, Matn2, and Igf2	Promotes CM proliferation and improves collagen deposition to protect cardiac rupture in the nonregenerative heart	Administration of Treg supernatant to CMs *in vitro*, cell depletion, data mining, and rescue experiments *in vivo*	Adult mice	**34**
	Treg – CMs	Ccl24-CCR, Gas6-AXL, Grn-EphA2, and Areg-EGFR	Supports heart regeneration	Cell depletion, NOD/SCID (T cell-depleted) mice, and scRNA-seq	Neonatal mice	**35**
Revascularization						
	epicardial cells – cECs	Cxcl12b – Cxcr4a	Promotes superficial sprouting of cECs in the injured epicardium of regenerating hearts	Genetic deletion	Zebrafish	**38**
	endocardium – cECs	Vegfaa	Drives intraventricular sprouting of cECs in regenerating hearts	Vegfaa gain-of-function (*flt1* mutant)	Zebrafish	**38**
	capillary cECs – arterial cECs	Cxcl12 – Cxcr4	Drives formation of collateral arteries to support regeneration	Genetic deletion	Neonatal mice	**39**
	cECs – cECs and epicardial-derived cells	Vegfc triggers *emilin2a* expression	Promotes CM dedifferentiation and proliferation in the regenerating heart	soluble Flt4 to block Vegfc signaling and genetic deletion	Zebrafish	**40**
	HUVEC – CMs		Overexpression of TFs in HUVECs stimulates proliferation of CMs *in vitro*	ATAC-seq, overexpression and co-culture of cells	Zebrafish and cell culture	**41**
Endocardium						
	ECs – CMs	Notch and Wnt signaling pathways	Drives CM proliferation in the regenerating heart	Endocardial-specific expression of DN-MAML, RNA-seq, and rescue experiments	Zebrafish	**43**
	ECs – immune cells	Notch	Restricts immune cell recruitment to the injured area in the regenerating heart	γ-secretase inhibitor to block Notch signaling, RNA-seq	Zebrafish	**42**
	ECs – neutrophils	Myd88, PI3K/AKT, and Cxcl18b	Drives neutrophil recruitment in the regenerating heart	Genetic mutant, scRNA-seq, RNA-seq, and overexpression	Zebrafish	**47**
Epicardium						
	macrophages – epicardium – endocardium – CMs	Vegfaa-Vegfr, Notch, and Nrg1-ErbB	Macrophage recruitment triggers epicardial *vegfaa*, driving endocardial *nrg1* and CM proliferation in the injured larval heart	RNA-seq, genetic macrophage ablation	Larval zebrafish	**50**
	macrophages – endo/epicardium	Tnfa – Tnfrsf1a	Regulates AP−1 expression driving key regenerative gene expression in endo/epicardium in the regenerating heart	scATAC-seq, cell type-specific genetic manipulation	Zebrafish	**52**
	Fb/epicardium – macrophages	Csf1 – Csf1r	*ptx3a*+ epicardial cells drive recruitment of Cxcr4b+ reparative macrophages in the regenerating heart	scRNA-seq data mining, genetic depletion, pharmacological inhibition	Zebrafish	**53**
CMs						
	Fb – macrophage	C3 – C3ar1	C3+ Fb recruit C3ar1+ macrophages in proximity to regenerative CMs in adult murine CMs overexpressing YAP	spatial transcriptomics, scRNA-seq, genetic mutant	Adult mice (YAP OE)	**54**
	macrophages – CMs	TNF, ADAM15, GDF, and IGF	C3ar1+ macrophages signal to CMs to promote CM regenerative hallmarks	spatial transcriptomics, scRNA-seq, genetic mutant	Adult mice (YAP OE)	**54**
	CMs – CMs	mechanotransduction signaling, YAP/TAZ pathway	Mechanical injury is sufficient to induce BZ-specific gene expression in CMs	single nuclei and spatial transcriptomics, scratch assay	Adult mice and cell culture	**58**
	CMs – CMs	BMP7 – BMPR1A/BMPR2A	Drives CM proliferation in adult murine hearts following MI and loss of *bmp7a/Bmp7* in zebrafish hearts/neonatal murine CMs reduces CM proliferation	Meta-analysis of RNA-seq, genetic mutant, recombinant protein injection	Zebrafish, neonatal/adult mice	**61**
Fb						
	platelets – macrophages – Fb	Cxcl4	Platelet-derived CXCL4 drives the activation of pro-fibrotic	scRNA-seq analysis, single nuclei RNA-seq, genetic mutant	Adult mice	**64**

(*continued on next page*)

Table 1 (*continued*)

	Cell-cell interaction	Signaling pathway involved	Impact on cardiac regeneration/repair	Technology used to describe the interaction	Model	Ref
			Spp1+ macrophages which activates Fb ECM signature in the nonregenerative adult murine heart			
	CMs – Fb	CCN1 – Trp53	Drives fibroblast senescence in the regenerating heart	Genetic mutant, AAV-driven gene knock-down	Neonatal mice	**65**
	Fb – CMs	Col12a1a+ Fb	Drives CM proliferation in the regenerating heart	scRNA-seq, genetic ablation	Zebrafish	**68**
ECM						
	ECs – CMs	Agrin-DAG1-YAP pathway	Drives hallmarks of CM regeneration in the nonregenerative adult murine heart	Mass spectrometry, genetic mutant, recombinant protein injection	Neonatal and adult mice	**69**
	ECs – Fb	Agrin-Integrin/FAK-ERK/ Akt1-Egr1 pathway	Promotes fibroblast senescence in regenerating neonatal and nonregenerative adult murine hearts	Proteomics and scRNA-seq	Neonatal and adult mice	**66**
	Fb – CMs	Versican-Integrin beta	Drives CM proliferation in regenerating neonatal and nonregenerative adult murine hearts	scRNA-seq, proteomics, genetic manipulation	Neonatal and adult mice	**71**
	macrophages – CMs	Osteopontin (SPP1)-CD44	Stimulates CM proliferation in the regenerating heart	Genetic mutant	Neonatal mice	**72**

highly conserved transmembrane receptors that regulate cell fate, whose large extracellular regions interact with neighboring Delta or Serrate/-Jagged family of ligands. The ligand-receptor interaction leads to cleavage of the Notch intracellular domain (NICD), which subsequently translocates to the nucleus, and together with its co-activator Mastermind-like (MAML) protein activates transcription of target genes [47,48]. Endocardial-specific inhibition of Notch signaling through expression of a dominant-negative mastermind-like (DN-MAML) protein in zebrafish hearts was shown to inhibit cardiac regeneration and scar resolution following ventricular amputation. Lack of Notch signaling in the endocardium led to non-cell autonomous effects on CM proliferation, likely through decreased expression of Wnt signaling pathway inhibitors *wif1* and *notum1b*, and Wnt pathway inhibition was able to partiallly rescue this CM proliferation defect when endocardial Notch signaling was inhibited [45]. Endocardial Notch signaling was also implicated in restricting the recruitment of inflammatory cells to the injured area during zebrafish cardiac regeneration, and global Notch inhibition through pharmacological treatment led to an increase in *l-plastin*+ leukocytes and *mpeg1*+ macrophages that contact the endocardium [44]. A recent study from Goumenaki and colleagues further emphasizes the interplay of the endocardium with innate immune cells during the regenerative response in zebrafish [49]. *myd88* null zebrafish hearts exhibit a decrease in recruited neutrophils following cryoinjury and this effect is partly mediated by the downstream chemokine gene *cxcl18a*. Notably, restoration of *cxcl18a* expression levels in *myd88* mutants or restoring *myd88* expression specifically in endocardial/endothelial cells can rescue neutrophil recruitment and fibrotic scarring phenotypes normally displayed by *myd88* mutants [49].

The epicardium is an epithelial-like cell layer, which forms the outermost layer of the heart, and its importance in zebrafish cardiac regeneration has been revealed by defects in CM proliferation and scar resolution when the epicardial cell layer is genetically ablated [50]. Several growth factors have been shown to arise from the epicardium in response to injury, and intercellular communication through ligand-receptor interactions likely contribute to the process of cardiac regeneration [51]. Recent studies have provided mechanistic insight into the interaction of epicardium, myocardium, and innate immune cells and how these interactions promote cardiac regeneration. For example, in a model of laser injury in larval zebrafish hearts, live-imaging analysis revealed the recruitment of diverse macrophage subtypes, and a contribution from macrophages in the removal of apoptotic CMs and stimulation of CM proliferation in response to injury [52]. Notably, macrophage recruitment to the injured heart occurs in the epicardial-myocardial niche and stimulates activation of *vegfaa*: EGFP expression in the epicardium, which was shown in the zebrafish heart to promote CM proliferation in response to injury [52,53]. In the adult zebrafish heart, interactions between immune cells and the epicardium have been recently shown to be an important regulator of heart regeneration. Mutation in tumor necrosis factor alpha (*tnfa*), whose expression is high in proinflammatory macrophages, results in a defect in cardiac regeneration following ventricular amputation [54]. Mechanistically, Tnfa from macrophages activates epicardial and endocardial cells through the Tnfrsf1a receptor following cardiac injury, and single-cell chromatin accessibility profiling revealed the enrichment of motifs from the downstream transcription factor AP-1 in open chromatin regions in both cell types [54]. Notably, blocking AP-1 through dominant negative approaches in epicardial or endocardial cells resulted in a block in regeneration and the downregulation of several target genes, including *postnb, col1a1b, and fn1a* in epicardial cells, and *fosl1a* and *raldh2* in the endocardium [54]. These results suggest a tight interplay between cytokine release from proinflammatory macrophages and the activation of the epicardium and endocardium to promote cardiac regeneration. On the other hand, fibroblast (Fb)/epicardial cells have recently been shown to recruit reparative macrophages to the heart during zebrafish regeneration [55]. Analysis of cell-cell communication from published scRNA-seq experiments revealed that outgoing signals from Fb/epicardium were mainly received by macrophages in the regenerating zebrafish and neonatal mouse heart. During zebrafish heart regeneration, a subset of *ptx3a*+ Fb/epicardial cells was enriched for the expression of genes involved in the immune response, and *ptx3a*+ cells were localized nearby reparative macrophages expressing *cxcr4b* and *mrc1b* [55]. Genetic depletion of *ptx3a*+ cells resulted in a decrease in *cxcr4b*+ macrophages, an increase in collagen deposition, and defects in scar resolution following injury. These effects were hypothesized to be mediated by the Csf1-Csf1r signaling axis, and pharmacological inhibition of the *csf1a* pathway resulted in similar phenotypes to *ptx3a*-ablated hearts [55]. Notably, the Csf1-Csf1r axis has also been implicated in a pro-regenerative niche present in adult murine hearts that overexpress constitutively-active Yes-associated protein (YAP) described in section 2.4 below [56]. Altogether, these results highlight the importance of endocardial and epicardial cells in orchestrating the regenerative response in other cell types in the heart, namely CMs and innate immune cells.

2.4. Cardiomyocytes

The Hippo signaling pathway is a central regulator of cardiac regeneration, and the transcription factor YAP has been shown in multiple contexts to stimulate CM proliferation, sarcomere disassembly, protrusion into the injured area, and functional recovery in the

nonregenerative mammalian heart [20,57–59]. A recent study has provided insight into how CM-specific overexpression of YAP can facilitate the formation of a pro-renewal niche comprised of macrophages and fibroblasts that promote cardiac regeneration in the adult mouse heart [56]. A combination of spatial and single-cell transcriptomic studies revealed increased presence of a fetal-like CM state, with upregulation of fetal genes and decreased expression of genes whose encoded proteins regulate oxidative metabolism, when constitutively active YAP5SA was expressed in CMs. Spatial interaction between regenerative CMs with complement ligand *C3*+ fibroblasts and complement C3 receptor *C3ar1*+ resident macrophages was shown in regenerative YAP5SA-expressing adult and injured neonatal murine hearts. Notably, *C3*$^{-/-}$ and *C3ar1*$^{-/-}$ mutant hearts exhibited defects in CM sarcomere disassembly and proliferation, cardiac function, and scar resolution that normally accompany YAP5SA-overexpression and neonatal heart regeneration. Ligand-receptor analysis of signaling pathways from *C3ar1*+ macrophages to CMs revealed potential regulation by TNFSF12-TNFRSF12A, ADAM15-ITGB1, and IGF1-IGF1R signaling to promote regenerative hallmarks in CMs. This elegant study highlights the importance of the complement signaling pathway and interactions between fibroblasts, macrophages, and CMs in promoting cardiac regeneration. Strikingly, while a small population of fetal-like CMs was found in the infarcted human heart, the presence of *C3*+ fibroblasts and *C3ar1*+ macrophages was not in close spatial contact to these cells, suggesting that all three cell types are essential for the generation of a pro-renewal niche to support regeneration [56].

CM-CM interaction and the transcription factor YAP has also been implicated in the transcriptional response of the border zone myocardium to injury [60]. In response to myocardial infarction (MI) in the adult murine heart, a "loss-of-neighbor" hypothesis was proposed whereby remaining CMs at the border zone elicit a rapid transcriptional response to mechanical destabilization resulting from cell loss in the ischemic region of the heart. This hypothesis was supported by single-nuclei and spatial transcriptomic methodologies revealing the upregulation of several genes, including *Ankrd1*, a YAP/TAZ target gene involved in mechanotransduction, in border zone myocardium. Further support for this hypothesis was provided by a similar transcriptional response to mechanical injury, including needle stab injury and scratch assays in neonatal rat ventricular myocytes (NRVMs), in the absence of ischemic cell death [60]. Transcriptional upregulation of *ankrd1a* in border zone myocardium is conserved in the regenerating zebrafish heart [61], and knockdown of *Ankrd1* in neonatal mouse hearts inhibited cardiac regeneration following apical resection [62], suggesting that the transcriptional response to mechanical stabilization may function to promote cardiac repair and regeneration.

CM-CM interaction through autocrine Bone morphogenetic protein 7 (BMP7) signaling has also been shown to promote cardiac regeneration [63]. Meta-analysis of RNA-sequencing datasets revealed a decrease in *Bmp7* expression in the neonatal murine heart at P10 (non-regenerative) compared to P1 (regenerative) stages, which was shown to be specific to CMs by RT-qPCR analysis. BMP7 administration to isolated CMs from P7 murine hearts and systemically to adult mice following MI stimulated CM cell cycle reentry. BMP7 administration also modestly decreased cell cycle entry of stromal cells in both isolated cells from P7 hearts and the adult murine heart following MI. *bmp7b* mutation in the regenerative zebrafish heart led to a decrease in CM proliferation following cryoinjury. These results suggest that CM autocrine signaling can stimulate CM proliferation in response to injury to promote cardiac regeneration [63]. Altogether, the studies described here suggest that CMs play an active role in regulating CCIs and intercellular crosstalk that promote cardiac regeneration.

2.5. Fibroblasts

Following cardiac injury, fibroblasts originating from the epicardium and endocardium form a highly heterogeneous group of cells that secrete various paracrine and ECM factors. Fibroblast differentiation into myofibroblasts is crucial for secreting ECM proteins that provide structural integrity and prevent heart rupture [64,65]. However, excessive myofibroblast activation can lead to acute fibrosis and increased heart failure risk, highlighting the need for careful balance of fibroblast activation. Recently, a population of pro-fibrotic *Spp1*+ macrophages in the adult murine heart were shown to promote cardiac fibrosis, and their expansion was correlated with human heart failure [66]. *Spp1*+ macrophage activation depends on platelet-derived CXCL4 and loss of *Cxcl4* results in decreased cardiac fibrosis in response to MI. *In vitro* co-culture studies revealed that *Spp1*+ macrophages that were stimulated by *Cxcl4*+ platelets can activate fibroblast ECM signatures and increase *Col1a1* and *Fn1* expression in fibroblasts [66]. This study demonstrates the interaction between platelets, macrophages, and fibroblasts in the heart and how this interaction can drive cardiac fibrosis in a nonregenerative adult heart.

The intricate balance between fibroblast activation and senescence has recently been shown to play an important role in heart regeneration. For example, secretion of Cellular communication network factor 1 (CCN1) from CMs in neonatal mouse hearts was shown to stimulate fibroblast senescence [67]. This senescence was essential for heart regeneration, as blocking senescence through the use of senolytic drugs, *Trp53* knockout, or *Ccn1* knockdown resulted in defects in CM proliferation and scar resolution [67]. Transient senescence in fibroblasts is driven by the Early growth response protein 1 (EGR1) and *Egr1* knockout results in a lack of cardiac regeneration in neonatal mouse hearts and adult mouse hearts treated with recombinant Agrin protein [68]. *Egr1* knockout led to a decrease in CM proliferation and neoangiogenesis in response to myocardial injury in the neonatal mouse heart, but whether these phenotypes are due to EGR1 function in these cells or fibroblast senescence was not tested [68].

Fibroblasts play an active role during heart regeneration in zebrafish and genetic ablation of *col1a2*-expressing cells has been shown to reduce CM proliferation [69]. scRNA-seq analysis at multiple timepoints following cryoinjury of the zebrafish heart revealed the diversity of fibroblast subsets deriving from the epicardium and endocardium [70]. Two populations of transient fibroblasts that arise in the injured heart are enriched for expression of genes encoding for secreted proteins, including *nrg1* and *fn1a*, that have been shown to promote CM proliferation. Notably, genetic ablation of *col12a1a*+ proregenerative fibroblasts resulted in decreased CM proliferation and defects in scar resolution in the cryoinjured zebrafish heart [70]. Altogether, these studies suggest that a population of proregenerative fibroblasts exists in the regenerative heart and that a fine balance between fibroblast activation and overactivation is likely necessary to stimulate cardiac regeneration and prevent cardiac fibrosis.

2.6. Extracellular matrix

The ECM is comprised of various extracellular proteins, including proteoglycans, structural proteins, enzymes, glycoproteins, and elastic fibers. While the primary role of the ECM is to maintain the structural integrity of tissue, increasing evidence suggests an important role in promoting regeneration of the heart. Decellularized zebrafish and neonatal P1 mouse cardiac ECM have been shown to stimulate CM proliferation *in vitro* [71,72]. Additionally, intramyocardial injection of zebrafish ECM increased CM proliferation and improved heart function in adult mice following MI [72], underscoring the potential of ECM to stimulate heart regeneration.

Recent studies have elucidated the mechanistic action of individual ECM components in promoting cardiac regeneration (Fig. 1B). Agrin, a heparan sulfate proteoglycan secreted by cardiac endothelial cells, is vital for neonatal mouse heart regeneration, and intramyocardial administration of recombinant Agrin promotes CM cell-cycle reentry and improves heart function in adult mice after MI [71]. Agrin binds Dystroglycan 1 (DAG1) to promote Extracellular signal-related kinase

(ERK) activation, and Agrin binding to DAG1 disrupts the dystrophin glycoprotein complex and leads to YAP nuclear translocation [71]. YAP localization in the CM nucleus was shown to promote several hallmarks of CM regeneration, as described in section 2.4. Recently, Agrin has also been shown to induce fibroblast senescence in the adult mouse heart, potentially through the Integrin/FAK-ERK/Akt1-Egr1 axis [68].

Another ECM component that was shown to promote heart regeneration is Versican, a matricellular proteoglycan that was found by scRNA-seq to be expressed in cardiac fibroblasts in neonatal mouse hearts 1 day post apical resection [73]. Treatment of neonatal mouse cardiomyocytes with recombinant Versican (VCAN) protein resulted in increased CM proliferation, and shRNA knockdown of Integrin-β1 abrogated the effect of VCAN treatment on CM proliferation. Fibroblast-specific deletion of *Vcan* in neonatal mice at P1 led to decreased CM proliferation and abrogation of cardiac regeneration, while intramyocardial injection of recombinant VCAN protein in adult mice improved CM proliferation and cardiac function following LAD ligation [73]. Altogether, this study highlights a fibroblast-CM CCI mediated by an ECM component that promotes cardiac regeneration.

Osteopontin (OPN, and also known as SPP1), secreted by macrophages following neonatal heart injury, was shown to play a crucial role in heart regeneration [74]. OPN-deficient neonatal mice exhibit impaired regeneration and intramyocardial injection of recombinant OPN into the ischemic zone of the nonregenerative adult murine heart following myocardial infarction promoted CM proliferation and infarct repair. Mechanistically, OPN stimulated CM proliferation through the CD44 receptor and increased YAP nuclear localization in neonatal CMs *in vitro*. OPN also facilitated the migration of neonatal CMs and non-myocyte cells, suggesting a broad regenerative effect [74]. OPN has previously been shown to be expressed in reparative macrophages in the heart in response to MI, and *Spp1* adult knockout mice were more prone to develop dilation of the left ventricle following MI [75]. However, expansion of an *SPP1*+ macrophage cluster was found in scRNA-seq analysis of cardiac tissue from human heart failure patients [66], illustrating that a delicate balance in the temporal dynamics or levels of OPN/SPP1 is necessary to stimulate cardiac repair and regeneration.

Altogether, these described studies on individual ECM factors underscore the importance of cell-ECM interaction in establishing a regenerative niche. Importantly, a single intramyocardial injection of several recombinant ECM molecules has been shown to stimulate cardiac repair and improve cardiac function in non-regenerative models, highlighting their potential therapeutic use in enhancing heart repair and regeneration in the adult human heart.

2.7. Extracellular vesicles as potential mediators of intercellular crosstalk during regeneration

EVs are a diverse group of cell-derived membranous structures originating from various cell types [76]. Initially thought to function primarily as a means for cellular waste disposal, EVs are now recognized as a significant mode of intercellular communication, potentially carrying proteins, metabolites, RNAs, and other molecules that contribute to normal cardiac function and cardiovascular diseases [76]. In zebrafish, heart injury alters the dynamics of EV production [77], suggesting a potential role for EVs in cell-cell communication that may facilitate heart regeneration. Notably, epicardial-derived EVs can promote CM proliferation in P1 and P7 mice following MI [78]. Treatment with epicardial EVs in P7 mice, however, does not lead to differences in scar size, basic composition of the scar, vascular density, or CM sarcomere disassembly, indicating that EV treatment is not sufficient for complete regeneration [78]. Notably, administration of EV cargo *miR-30a*, *miR-100*, and *miR-30e* was sufficient to induce human CM proliferation and recovery of cryoinjured engineered human myocardium, suggesting a role for EVs in the exchange of miRNAs to stimulate CM proliferation and repair [78]. As the EVs in this study were derived from epicardial cells cultured *in vitro*, the potential for epicardial EVs derived from regenerating hearts *in vivo* to stimulate heart regeneration is not yet known. More studies are necessary to understand whether EVs drive intercellular communication that promotes heart regeneration.

3. Technical strategies to investigate cell-cell interactions in the heart

Technologies to investigate CCIs in finer detail have recently emerged (reviewed in [1,2,79,80]). Below, we focus on technical methods that have been applied in the heart or represent promising strategies to explore CCIs during cardiac regeneration *in vivo* (Fig. 2).

3.1. Live imaging

Optical microscopy is a methodology to observe and infer CCIs, and has been combined with histological/immunofluorescence staining, *ex vivo* culture, and electron microscopy. Live imaging has been utilized in zebrafish and mouse to observe early-stage heart tube development [81] and endocardial protrusions during cardiac trabeculation [82]. During cardiac regeneration in zebrafish, live imaging in whole ventricles or *ex vivo* tissue slices has provided important insight into cardiovascular EVs [77], immune cell recruitment [83], revascularization [84], and CM dynamics upon injury [19,85]. These insights have generated hypotheses about the role of CCIs in cardiac regeneration that could potentially be tested using methodologies described below.

3.2. Transcriptomic-based strategies

Computational tools have advanced the use of single-cell and spatial OMICS data to predict and identify ligand–receptor interactions based on different mathematical models that are actively undergoing iteration to improve prediction ability [80,86,87]. Considerable ligand-receptor interactions have been identified by computational analysis in the human heart [88,89]. However, alternative approaches may provide more precise information about CCIs and their function. For example, two methodologies have combined scRNA-seq of physically interacting cells, obtained through microdissection or incomplete tissue dissociation, and deconvolution of these interacting cells by comparison to a reference scRNA-seq of each cell type. ProximID was shown in adult murine bone marrow and intestinal crypt tissue to report novel preferential CCIs in both tissues, which was verified by single molecule fluorescence in situ hybridization [90]. Similarly, PIC-seq was applied in mouse embryos, neonatal murine lung tissue, and adult lymph node, using calibrated tissue dissociation protocols and the use of cell type-specific fluorescent transgenic lines. Fluorescence activated cell sorting (FACS) was used to isolate doublets of two specific cell types (with two different fluorescent reporters) or to isolate one cell type (marked with a fluorescent reporter) and distinguish cell doublets based on size. PIC-seq was able to detect novel CCIs and predict gene regulatory networks that are activated in response to CCI [91,92]. Caveats to these methodologies include bias towards strong CCIs that withstand the tissue dissociation protocol, the use of fluorescent transgenic lines to isolate cell doublets by FACS, and the necessity of sophisticated bioinformatic algorithms to deconvolute cell type-specific gene expression from doublets. However, elucidating gene expression networks that can be predicted from cells that physically interact has the advantage that informed hypotheses can be generated to investigate the role of CCIs and tested via genetic/pharmacological means to understand their importance in a process such as cardiac regeneration.

3.3. Synthetic strategies to investigate CCIs

Synthetic strategies have recently emerged to capture neighboring cells for further visualization and gene expression analysis [2]. For example, uLIPSTIC, a universal version of LIPSTIC (labelling immune partnerships by SorTagging intercellular contacts) has been recently

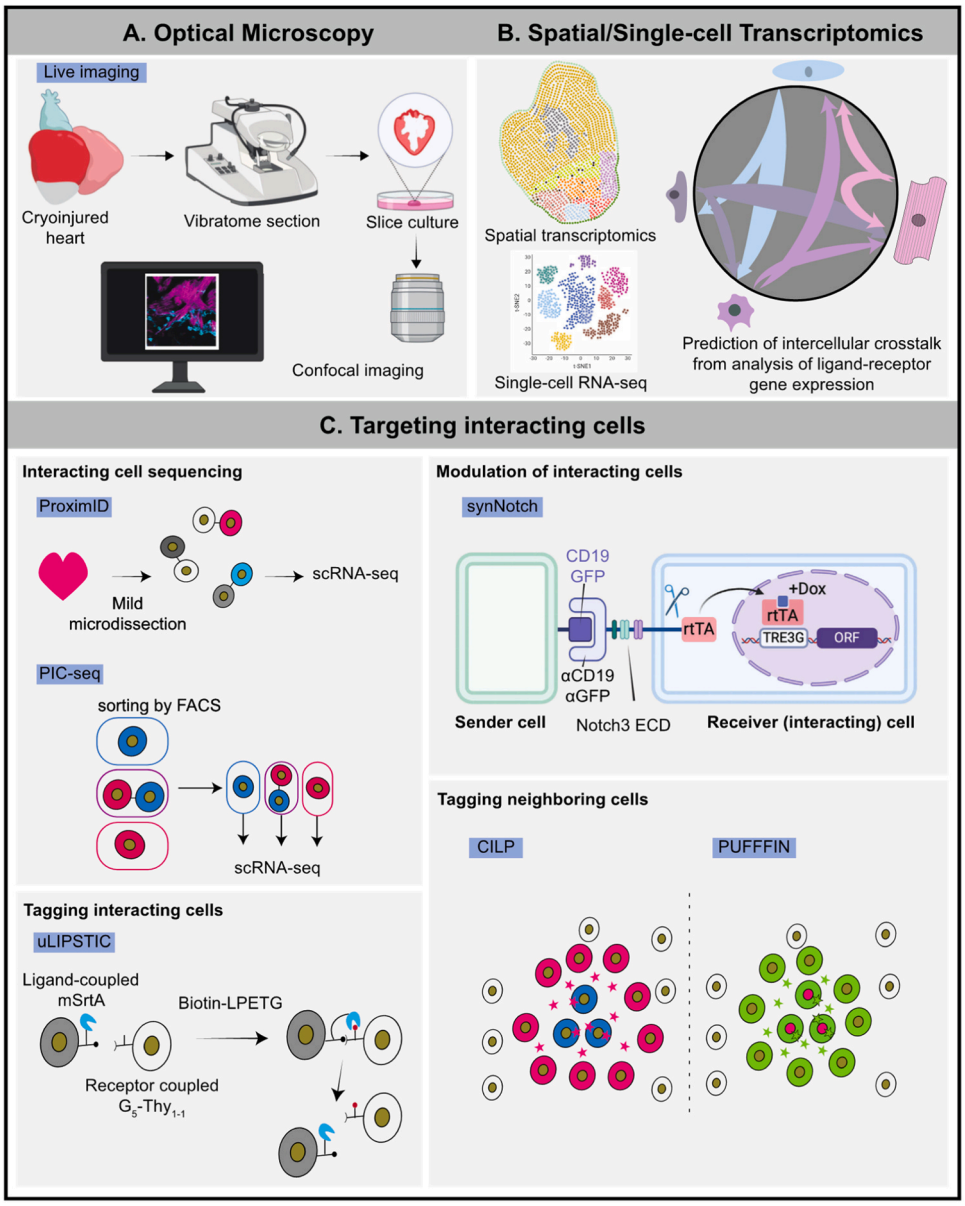

Fig. 2. Technologies to investigate cell-cell interaction and cellular crosstalk in the heart.**(A)** Schematic illustrating the live imaging technique that has been used to investigate zebrafish heart regeneration. **(B)** Schematic illustrating the use of spatial transcriptomics and single-cell RNA-sequencing to infer intercellular crosstalk using bioinformatic algorithms. **(C)** Emerging technologies to investigate or modulate cell-cell interaction and neighboring cells. PIC, physically interacting cells; uLIPSTIC, universal labeling immune partnerships by SorTagging intercellular contacts; synNotch, synthetic Notch; CILP, Cre-induced intercellular labeling protein; PUFFFIN, positive ultra-bright fluorescent fusion for identifying neighbors; ECD, extracellular domain; rtTA, reverse tetracycline-controlled transcriptional activator; Dox, doxycycline; TRE3G, Tet-responsive element third generation; ORF, open reading frame.

developed, utilizing the ability of extracellular membrane-tethered Staphylococcus aureus transpeptidase sortase A (mSrtA) to covalently transfer a labeled LPETG peptide motif onto an amino-terminal penta-glycine (G_5 fused to the protein $Thy_{1.1}$, G_5-$Thy_{1.1}$) acceptor [93]. In the published study, a loxP-flanked G_5-$Thy_{1.1}$ open reading frame followed by a downstream mSrtA coding sequence was inserted into the *Rosa26* locus for ubiquitous expression. Cre-mediated recombination leads to switching of the transgene from the receiver state to a donor state, allowing for readout of cell type-specific CCIs from whole tissue. uLIPSTIC allows visualization and droplet-based scRNA-seq for quantitative interaction-based transcriptomics [93]. One advantage of this system is the historical readout of CCI, as any cell that has interacted with the sender cell at any timepoint will be covalently modified and available for further study.

To visually label cell neighbors, Cre-induced intercellular labeling protein (CILP) has been developed by fusing lipid-soluble and membrane permeable peptides (HIV-1 transactivator of transcription, TATk) together with mCherry, to reveal juxtaposition of regenerative hepatocytes and liver endothelial cells in mice *in vivo* [94]. Another cell neighbor-labeling system has also recently emerged, termed Positive ultra-bright fluorescent fusion for identifying neighbors (PUFFFIN). In this system, secreted mNeonGreen- or HaloTag-fused positively supercharged fluorescent protein s36GFP is taken up by cell neighbors, which facilitates ultra-bright, sensitive, color-of-choice labeling to visualize or isolate cells nearby a specific cell type of interest [95]. Advantages of the CILP and PUFFFIN systems include the relatively simple transgene construction, but one limitation is that CCIs are only transiently labeled.

By artificially engineering the Notch-Delta signaling cascade, Morsut *et al.* presented a synthetic Notch (synNotch) receptor to read out CCIs. The system functions by utilizing the intracellular domain of Notch

coupled to a transcriptional regulator, which is activated by intramembrane proteolysis when induced by the engagement of a cognate extracellular ligand on the cell membrane [96]. Despite requiring complex plasmid construction and transgenic animal generation, synNotch provides various possibilities of customized downstream functional responses in neighboring cells depending on the transcriptional regulator coupled to the intracellular domain of Notch. Utilizing the synNotch system, it was shown that endothelial cells in the developing heart migrate and contribute to the liver vasculature, and that cardiac endothelial cells neither exhibit hemogenic potential nor contribute to cardiac macrophages or other circulating blood cells [97,98]. Of note, additional advantages and limitations of synNotch have been comprehensively discussed in a previous review [2].

4. Inter-organ communication

It has become increasingly clear that in addition to CCIs, inter-organ communication plays a crucial role in the response to cardiac injury. In 2019, it was shown that CM polyploidization, which negatively affects cardiac regenerative ability in zebrafish and adult mammalian hearts [99,100], was negatively correlated with acquisition of endothermy and levels of circulating thyroid hormone [101]. Notably, CM-specific abrogation of thyroid hormone signaling promoted CM proliferation and improved cardiac function in the adult mouse heart, whereas treatment of adult zebrafish with exogenous thyroid hormone disrupted cardiac regeneration following ventricular resection [101]. A recent study has further shown that Triiodothyronine (thyroid hormone T3) treatment induces a pro-inflammatory Ly6C^{high} monocyte/macrophage profile in injured neonatal murine hearts, leading to decreased CM proliferation and a block in cardiac regeneration [102]. These studies suggest that the levels of thyroid hormone, and crosstalk between the thyroid gland and heart, negatively affect the regenerative capacity of the heart.

The nervous system has also been shown to affect the regenerative ability of the heart. For example, genetic models of cardiac hypoinnervation in zebrafish exhibit decreased CM proliferation and defects in scar resolution following ventricular resection [103]. Further, inhibition of cholinergic nerve transmission through pharmacological and mechanical means led to an abrogation in regenerative ability in the neonatal murine heart [103]. In line with these results, α1-adrenergic signaling has recently been shown to regulate neuro-immune crosstalk and establish a regenerative microenvironment to promote cardiac regeneration in zebrafish. Blocking α1-adrenergic signaling specifically in macrophages led to changes in the phenotypic profile of cardiac macrophages, and resulted in decreased vascular density and CM proliferation following cryoinjury [104]. Counterintuitively, local sympathetic denervation in the adult mouse led to decreased inflammatory cell infiltration and CM hypertrophy, and improved cardiac function following MI [105]. These results suggest that neuronal-cardiac interactions impact cardiac regenerative ability, but that these interactions are likely context-dependent.

A recent study has also shed light on how cardiac injury can affect responses in distant organs. Genetic ablation of CMs in zebrafish results in an upregulation of the CCAAT enhancer binding protein delta (*cebpd*) gene locally in the heart, but also in distally in the brain and kidney. This response was shown, at least in part, to arise from alterations in fluid homeostasis and was regulated by corticosteroid receptors in the brain [106]. The heart-brain-kidney axis was previously shown to impact the adaptation to cardiac stress [107] and there is a well-known co-occurrence of heart failure with kidney dysfunction [108], highlighting the importance of understanding the role of inter-organ communication in both cardiac regeneration and disease settings.

5. Conclusions and perspectives

The goal of cardiac regenerative therapies is to replace injured tissue with cardiomyocytes to restore the function of the heart. From studies in regenerative organisms such as zebrafish, neonatal mice, and salamander, it is clear that intercellular crosstalk in the injured heart is essential for the establishment of a regenerative niche to promote cardiomyocyte dedifferentiation, proliferation, and replenishment of fibrotic tissue. With the rapid development and refinement of imaging, transcriptomic, and synthetic strategies to investigate and manipulate CCIs in the heart, we stand poised to investigate these interactions with unprecedented detail in the regenerative heart. Understanding the downstream gene regulatory networks activated in response to CCIs can inform the development of therapeutic strategies to establish a microenvironment in the heart to promote regeneration and prevent maladaptive remodeling of the human heart in response to injury and disease.

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