


## Talkin' 'bout regeneration: New advances in cardiac regeneration using the zebrafish

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### Abstract

The adult human heart has a very poor capacity to repair itself following injury. During heart attack, an enormous amount of cardiac tissue is lost from ischaemia. Whilst a low level of proliferation exists within the heart, the rate is insufficient to restore what is lost following ischaemic injury. In contrast to mammals, the zebrafish can completely grow back its heart following injury. This discovery, almost two decades ago, has resulted in something of a renaissance in the study of cardiac regeneration. Using the zebrafish, study has moved from observation of the phenomenon, to the application of different injury methods, tracing the origin of regenerated tissue, analysis of the different cellular contributions to regeneration and ongoing investigations onto the genetic cues that instruct the repair process (Figure 1). Progress has been considerable and provides us with important insights into a process we hope to one day apply to the injured human heart.

### Introduction

The heartbeat is so essential for survival, it is often used as the indicator of life. Cardiomyocytes are the cellular units of cardiac muscle and are responsible for contraction of the heart. It is estimated that up to a billion cardiomyocytes can die following a heart attack (myocardial infarction) due to ischaemia [1]. This loss of cardiomyocytes impairs cardiac output and can result in heart failure – an enormous medical and economic burden and one of the most common causes of death world-wide [2]. For this reason, there is considerable attention directed towards improving recovery from myocardial infarction.

One of the major contributing factors to the morbidity and mortality rate associated with myocardial infarction is that, in mammals, cardiomyocytes have a limited capacity to proliferate beyond neonatal stages [3]. In the neonatal mouse, cardiac injury is resolved by complete regeneration of the lost tissue [4]. Isolated clinical cases suggest that a similar regenerative

potential also exists in humans at neonatal stages [5]. In both mouse and man, however, this capability is lost in adulthood and, instead of repair, fibrosis ensues [6]. What limits this proliferative potential in adult mammals, why fibrosis develops instead of regeneration occurring, and why these limits are not imposed in certain species is an area of intense focus.

Unlike mammals, the zebrafish is capable of regenerating its heart upon injury as an adult [7]. This fact, and the ease of husbandry and genetic amenability, has meant this animal has emerged as a popular model organism for the study of cardiac regeneration. Whilst improving cardiomyocyte proliferative potential is a focal point in cardiac regeneration, it is clear that a symphony of cellular and extracellular cues are activated and essential for regeneration (Figure 1). In this review, we briefly summarise what is known in the field and emphasise the most recent advances in cardiac regeneration using the zebrafish model.

### **The regenerative response to cardiac injury in the zebrafish**

The capability of the zebrafish heart to regenerate was first described in a landmark study conducted by Poss, Wilson & Keating in 2002 [7]. In this work, approximately 20% of the ventricle was resected and the repair response followed over time. Following profuse bleeding in the seconds after injury, a clot consisting predominantly of erythrocytes formed and persisted for several days. From 2 days post injury (dpi), the clot was progressively replaced by deposition of fibrin and from 9 days onward, cardiomyocytes grew into the wounded area (driven by proliferation), replacing the fibrin matrix. By 60 dpi, the ventricular wall was replenished and apparently completely recovered.

Other methods to induce cardiac injury have since been used, including genetic ablation and cryoinjury [8–10]. Cryoinjury has risen to the fore as the method-of-choice, perhaps due to the reproducibility and ease of this method but also because this approach models certain aspects of myocardial infarction that other methods do not: cryoinjury causes damage to a localised region of the heart and it also leaves behind necrotic and apoptosing tissue, that must then be cleared away as part of the repair response. These aspects result in a more prolonged repair process, whereby cryoinjury takes more than 130 days to resolve [9,10].

The contrast between the capability of the zebrafish heart to regenerate and the failure of the mammalian heart to do so has been attributed to an insufficiency of cardiomyocyte turnover in mammals and a stark difference in the injury response: following cardiac injury and fibrin deposition, the mammalian heart responds with fibrosis or scarring. That is, the replacement of the fibrin matrix with collagen, fibroblasts and other non-contractile components filling the void of what was occupied substantially by cardiomyocytes [6]. The non-contractile scar

results in reduced cardiac function and, when it affects a large enough region, culminates in heart failure. The observation that the zebrafish heart does not become fibrotic but, instead, undergoes repair has captivated researchers and prompted intense investigation into the cellular and molecular mechanisms that are responsible for regeneration.

### **Multinucleated or polyploid cardiomyocytes have reduced proliferative capacity**

In both the neonatal mouse and zebrafish adult heart, successful cardiac regeneration is dependent on the generation of new cardiomyocytes and these regenerated cardiomyocytes derive from the proliferation of pre-existing cardiomyocytes [11–13]. One commonality between cardiomyocytes in these two contexts that differs from the adult mouse heart is their state of ploidy: The adult mouse heart consists largely of binuclear or polyploid cardiomyocytes whereas zebrafish and mouse neonatal hearts are predominantly mononuclear and diploid in nature (Figure 2) [14]. Whilst a correlation between the regenerative potential and ploidy state of cardiomyocytes had been noted [15], causality was only recently tested. Cardiomyocyte polyploidy was induced by the overexpression of Tnni3k or transient expression of a dominant-negative (dn) Ect2 protein (both of which increased DNA content, without inducing cytokinesis). These perturbations, resulting in polyploidisation of cardiomyocytes, was shown to be sufficient to impair cardiac regeneration in the zebrafish (Figure 2) [16,17]. Intriguingly, polyploidy does not impair cell proliferation in other contexts, including mammalian liver [18], *Drosophila* epithelium [19] or *Drosophila* intestine [20,21], it is therefore unclear why cardiomyocyte proliferation specifically is impacted. Interestingly, polyploidisation by either Tnni3k or dnEct2 overexpression prevented not only cytokinesis but also prevented an increase in DNA content (as determined by PcnA and BrdU staining, respectively), suggesting the impact of polyploidisation is not restricted to cell division. One theory put forward is that cardiomyocytes have a limited number of cytokinesis events possible in their lifetime and polyploidisation may contribute to exhausting this limit [16]. It is an intriguing possibility, one that gain-of-function models exhibiting excessive cardiomyocyte proliferation might be positioned to address [22,23].

During evolution, the development of polyploid cardiomyocytes has been shown to correlate with the switch to endothermy, warm-bloodedness and increased serum thyroid hormone levels. To investigate a causal relationship in this observation, inhibition of thyroid hormone signalling was tested for its impact on cardiomyocyte polyploidisation. Inhibition corresponded with a decrease in polyploid cardiomyocytes and improved regenerative response. Reciprocally, increasing thyroid hormone levels in normally regenerating *Xenopus* resulted in increased binucleation of cardiomyocytes and scar formation following injury [24,25]. Sitting somewhat at odds with these observations is that a lack of regenerative ability can also be

found in cold-blooded fish such as the medaka [26,27] and Mexican cavefish [28]. This suggests that additional mechanisms underlying the ability to regenerate exist or that these species are exceptions to the rule.

### **Growth factor signalling is important during regeneration for cardiomyocyte proliferation**

Whilst the state of the cardiomyocyte nucleus and its effect on proliferation will no doubt continue to be an area of active research, investigations into the signalling pathways that promote cardiomyocyte proliferation have for many years attracted considerable attention (for summary see Table 1). One signalling pathway that has emerged as a major player in cardiomyocyte proliferation is that of the Neuregulin1 (Nrg1)-ErbB pathway. Nrg1-ErbB has been identified as a potent mitogenic pathway enhancing cardiomyocyte proliferation in fish, mouse and rat [22,29,30]. Nrg1 expression is induced by cardiac injury, is required for the regenerative response and overexpression can enhance cardiomyocyte proliferation during regeneration, suggesting that its expression level may be rate-limiting in cardiac regeneration. Interestingly, overactivation of Nrg1-ErbB for prolonged periods results in cardiomegaly due to hyperproliferation, indicating that controlled administration of Nrg1 will be required if it is to be harnessed as a therapeutic agent. The key role this pathway plays in cardiomyocyte proliferation was recently highlighted following a drug screen for factors that promote cardiomyocyte proliferation [31]. Unexpectedly, vitamin D was identified for promoting proliferation during both development and regeneration of the zebrafish heart. Constitutive activation of the vitamin D receptor induced cardiomyocyte proliferation and, like Nrg1-ErbB, prolonged activation resulted in cardiomegaly. Surprisingly, these effects could be prevented by pharmacological inhibition of the Nrg1-ErbB pathway, demonstrating that vitamin D promotes cardiac hyperplasia by signalling upstream of the Nrg1-ErbB pathway.

A number of members of the TGF $\beta$  superfamily have also been implicated in altering cardiomyocyte proliferation following cardiac injury. The BMP pathway was identified using a spatially resolved transcriptomics approach on cryoinjured zebrafish hearts. Enrichment for components of the BMP signalling pathway was identified in regenerating hearts. By transiently inhibiting BMP signalling using a heat-shock-inducible noggin transgenic line, regeneration was impaired. Reciprocally, activating the pathway using an inducible BMP2b transgenic line enhanced regenerative capability, demonstrating that BMP signalling is necessary for cardiac regeneration and promotes cardiomyocyte proliferation [32]. The TGF $\beta$  ligand, *Inhbaa*, has also been shown to promote cardiomyocyte proliferation and transgenic

overexpression results in a faster resolution of the wound compared with wildtype controls following cryoinjury [33].

### **Transcriptional control of cardiomyocyte proliferation during regeneration**

Ultimately, these signalling pathways must be interpreted into a transcriptional response that activates the cell cycle. Transcriptomic analysis of the border zone shows exactly this, with upregulation of genes involved in cell cycle and mitosis observed [32,34]. Interestingly, there appears to be a concomitant downregulation of cytoskeletal gene expression in regenerating cardiomyocytes. It has been appreciated for some time that cardiomyocytes undergoing proliferation disassemble their sarcomeres and loosen cell adhesions in preparation for cell division [35]. This regulation appears to extend to transcriptional control of these structural proteins, whereby sarcomeric proteins and cytoskeletal components are down regulated in proliferating cardiomyocytes. This orchestrated silencing appears to be regulated epigenetically, as many of the structural components are labelled by H3K27me3 repressive marks. Alleviating this repression by sequestration of the polycomb repressive complex, PRC2, increased the expression of a range of sarcomeric proteins. Interestingly, this increase in structural proteins coincided with impaired cardiac regeneration, suggesting that the mere concentration of these structural components is sufficient to inhibit cytokinesis.

In terms of transcriptional regulation, it is intriguing that a range of different genetic programs are activated by cardiac injury. Not only is gene expression induced upon injury but in a spatially restricted manner – such as induction at the “border zone” (the region adjacent to the injury site). A regulatory mechanism for this phenomenon was recently reported by the examination of enhancer elements activated only in the heart in response to injury [36,37]. Genome-wide profiling identified “CREEs”: cardiomyocyte-specific regeneration enhancer elements. Conceptually, these enhancer elements exist to engage transcription factors in response to tissue damage to regulate genetic programs for regeneration. Further work into these CREEs may shed further light on the epigenetic programs controlling myocardial proliferation [38]. It's also tempting to imagine that these elements could be engineered to boost the regenerative potential of mammalian organs.

Neural crest cells are a highly migratory population of cells that derive from ectoderm and give rise to melanocytes, cartilage and bone, smooth muscle cells and neurons. It has been a longstanding question as to whether the neural crest contributes cells to the myocardium of the zebrafish heart during development. Recent evidence suggests this is indeed the case, whereby lineage analysis in chick, mouse and zebrafish demonstrate this contribution [39] (<http://dx.doi.org/10.1101/6625360>). Surprisingly, a subset of cardiomyocytes start to express

neural crest regulatory network genes, including sox10, after injury [39]. These sox10-positive cardiomyocytes are important for heart regeneration as genetic ablation of the population inhibits regeneration (<http://dx.doi.org/10.1101/6625360>).

### **The contribution of the endocardium to cardiac regeneration**

The endocardium serves as the interface between the myocardium and luminal blood flow. The endocardium is a mechanosensitive tissue and, in response to blood flow, signals to the myocardium for correct development. It also performs this role in the context of cardiac injury, responding to disturbed flow and activating signalling from endocardium to myocardium. Signalling mechanisms, to date, have been traced to operate through the Notch pathway. Notch signalling is upregulated in the endocardium upon injury and this upregulation is required for cardiac regeneration [40,41]. Recently, this response was demonstrated to be mediated by altered hemodynamic forces that occur following cardiac injury. This altered flow is detected by Trpv4, a mechanosensitive channel expressed in the endocardium. Trpv4 activation coincides with the expression of the mechanoresponsive transcription factor, Klf2a, which in turn activates endocardial Notch signalling [42].

Notch signalling appears to act as a key signalling node, whereby it functions upstream of Nrg1-ErbB, Wnt and Bmp signalling. Both Nrg1-ErbB [40, 41] and Bmp pathways [42] promote cardiac regeneration and Notch signalling positively regulates the activity of these pathways. Wnt signalling, by contrast, has been shown to be a negative regulator of cardiac regeneration and Notch acts to repress Wnt activity [43]. In the absence of Notch repression, cardiomyocyte proliferation decreases and fibrosis occurs. Reciprocally, pharmacological inhibition of Wnt increases cardiomyocyte proliferation and can rescue, to a large extent, the diminished cardiomyocyte proliferation observed under Notch loss-of-function conditions. Interestingly, all three pathways function in the myocardium, whereas Notch signalling is active in the endocardium. How this non-cell-autonomous signalling to the myocardium by Notch is achieved has not yet been established.

As well as a direct role in signalling during regeneration, the endocardium contributes endothelial cells that comprise the coronary vasculature [44]. Just 15 hours after cryoinjury, angiogenic sprouts can be observed growing into the wound site [45]. These sprouts are hypothesised to derive from pre-existing coronary vessels and are not contributed by Tcf21-positive epicardial cells. This rapid vascular growth is regulated by Vegf signalling and is necessary for cardiomyocyte proliferation and regeneration.

### **Multinucleated cells at the leading edge assist epicardial regeneration**

The epicardium forms the outer layer of the heart and, like the endocardium, is activated after injury. The activated epicardium is an important source of factors that are essential for proliferation and survival of cardiomyocytes as well as a source of cells, including perivascular cells and fibroblasts, that are contributed to the wound during heart regeneration (reviewed in detail in [46]). Recently, it was shown that the epicardium regenerates as a large sheet 'or wave' of cells. Within this sheet, cells are specialised to contribute to regeneration by either migration or cell renewal, based on their location. The wavefront consisted of large, multinucleated leader cells and mechanical tension on these cells, which was shown to be highest in leader cells during migration, was sufficient to induce endoreplication. It is supposed that the multinucleation allows the cells to become larger, permitting them to cover a greater surface area and more effectively regenerate the epicardial surface. Behind the leader cells, the aptly named follower cells were described that divide to produce small, mononuclear daughters through cytokinesis to increase cell number [47]. The regenerating epicardium has also been shown to express Neuropilins (Nrp1a). Nrp1a loss-of-function results in a significant delay in heart regeneration with reduced vascularisation and epicardial-derived cell migration [48].

### **The timing and types of immune cells at the wound are crucial in preventing scar formation**

Inflammation plays a crucial role in cardiac regeneration, and only in the last few years are we starting to understand more about how the immune response tightly controls the difference between regeneration and fibrosis or scarring. Immediately after injury, inflammatory cells are recruited to the site of injury to clear debris and dead cells. In addition to their clearance function, these cells also express cytokines that are responsible for attracting (myo)fibroblasts to the wound site. Over time, different populations of neutrophils, macrophages, eosinophils and T-cells are recruited [49]. Similar to the human situation, different macrophage subsets are found with inflammatory Tnf $\alpha$ -positive macrophages promoting scar deposition and Tnf $\alpha$ -negative macrophages facilitating scar removal during regeneration. Osteopontin was found to control inflammatory cell resolution, but also drive scar formation. Comparing zebrafish with medaka, the latter of which do not regenerate their hearts after injury, also highlighted the impact of the timing of recruitment of the different leukocyte populations, the balance between these populations and the time taken to depart the injury site on heart regeneration [27]. A major difference between these fish species during regeneration was identified in their immune response. Macrophage recruitment was found to be delayed and reduced in medaka compared with zebrafish. A delay in neutrophil clearance was also observed, resulting in neutrophil persistence at the wound. Depletion of macrophages at the onset of cryoinjury in zebrafish compromised neutrophil clearance, emulating the response in medaka. This

coincided with reduced vascularisation of the wound and reduced regeneration. Reciprocally, stimulating Toll-like receptor signalling in medaka enhanced neutrophil clearance and promoted wound healing [27].

Excessive leukocyte accumulation in the injured area has also been shown to impair cardiac regeneration as observed in the zebrafish breakdance (*bre*) mutant. Importantly, this impaired regenerative response can be rescued by administration of mild dosages of anti-inflammatory or prokinetic drugs [50]. Mexican cavefish, like medaka, do not regenerate and have markedly different immune response to the zebrafish. Not only do they show extensive upregulation of the innate immune system but also their adaptive immune response is increased [28]. In addition to the roles described above, Notch and Nrg1 signalling have also been shown to influence the immune response. Regulatory T-like cells (zTreg) stimulate regeneration through interleukin-10-independent secretion of Nrg1 and conditional ablation of these cells inhibits regeneration. Recombinant Nrg1 is able to rescue the regeneration defects associated with zTreg cell depletion, whereas *Foxp3a*-deficient zTreg cells infiltrate but fail to express regenerative factors, such as Nrg1[51]. Notch signalling inhibition results in increased expression of the pro-inflammatory gene *tnfrsf9a* in the wound endocardial cells and elevated numbers of macrophages associated with wound endocardial cells [52].

### **Future perspectives**

Whilst headway has been made into understanding the cellular and genetic requirements that enable a zebrafish heart to regenerate, there is still much to uncover about the process. New directions into deciphering the mechanisms underlying zebrafish heart regeneration has recently been shown for the zebrafish cardiac valves [53]. In preprints the role the metabolic status of cardiomyocytes plays in regeneration is being explored (<https://doi.org/10.1101/498899>), as well as the role of *runx1* and myofibroblast gene expression in the wound in unexpected cell populations, including thrombocytes (<https://doi.org/10.1101/799163>). These provide clear examples that we do not yet have to all the answers we may need to unlock any regenerative potential in a clinical setting. This is hampered further by considerable differences that exist between zebrafish and humans, demanding caution in interpreting and translating these findings. Importantly, several zebrafish discoveries have been substantiated or found consistent in the mammalian context, providing hope and confidence that we are on the right track.

### **Conflict of interest**

The authors have no conflicts to declare



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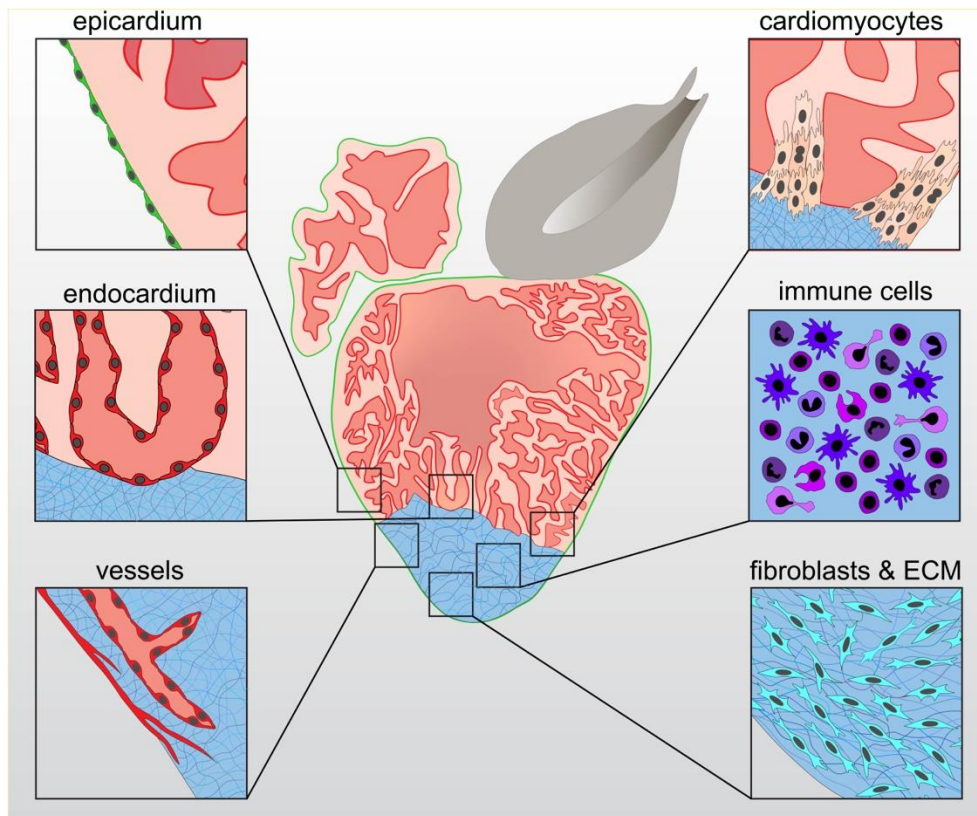
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This study identified zebrafish T<sub>reg</sub> cells and showed they home to the site of injury in a range of damaged organs, including the heart. Ablation of T<sub>reg</sub>s impaired the regenerative response and genetic ablation of *foxp3a* impaired T<sub>reg</sub> cell ability to secrete regenerative factors (such as Nrg1, in the case of the heart). Interestingly, *foxp3a* ablation does not impair T<sub>reg</sub> cell homing to the injury site and yet cardiac regeneration is impaired. Supplementation of Nrg1 was sufficient to restore cardiac regeneration when T<sub>reg</sub>s were ablated.

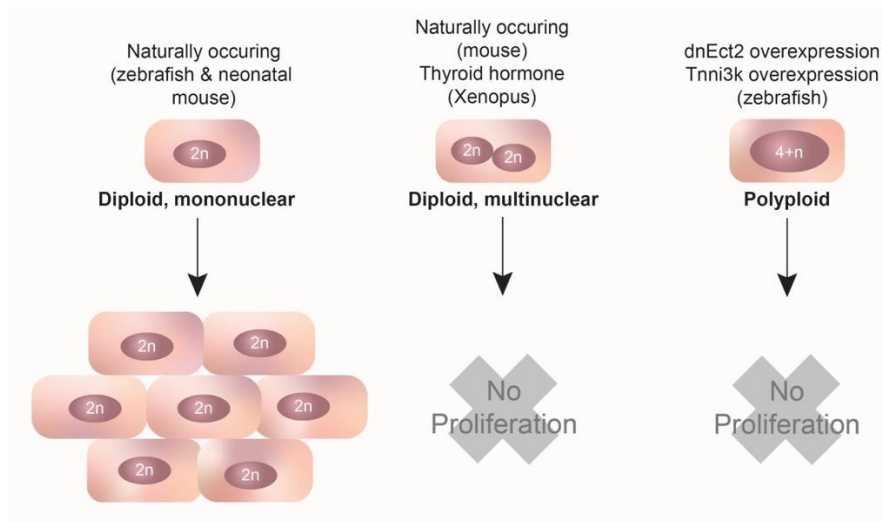
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**Figure 1. Schematic of cell types that contribute to the regenerating zebrafish heart.** Upon injury, the zebrafish heart deposits ECM at the site of injury (blue region). Regeneration occurs through proliferation of existing cardiomyocytes (pink), regeneration of the epicardium (green) and signalling from both epicardium and endocardium (red) occurs, vascularisation of the wound is rapid and fibroblasts (cyan) and ECM are found in the wound. Immune cells (purple) also infiltrate the wound, clearing away debris and signalling to cardiomyocytes.



**Figure 2. The number of nuclei and state of ploidy impact on cardiomyocyte proliferation.** Cardiomyocytes require to be in a mononuclear, diploid state for cytokinesis to occur. Multinucleation (including binucleation) of cardiomyocytes is more predominant in particular mouse strains and is promoted by increasing serum thyroid hormone levels in Xenopus. Polyploidisation can occur by inhibiting Ect2 (by overexpressing a dominant-negative [dn] form of Ect2) or by overexpressing Tnni3k. Upon multinucleation or polyploidisation, cardiomyocyte proliferation is impaired.

**Table 1.** Summary of genetic and signalling factors known to impact regeneration upon modulation of expression or activity

<b>Factor &amp; perturbation</b>	<b>Impact on regeneration</b>	<b>Source of expression</b>	<b>Site of action</b>	<b>Ref</b>
Dominant-negative Ect2 overexpression	Impaired	Cardiomyocytes	Cardiomyocytes	16
Tnni3k overexpression	Impaired	Cardiomyocytes	Cardiomyocytes	17
Neuregulin1 overexpression	Improved	Cardiomyocytes and Regulatory T cells	Cardiomyocytes	29, 40, 41, 51
ErbB (neuregulin receptor) activation	Improved	Cardiomyocytes	Cardiomyocytes	29, 40, 41,
Inhbaa overexpression	Improved	Cardiomyocytes	Cardiomyocytes	33
Wnt signalling activation	Impaired	Cardiomyocytes	Cardiomyocytes	43
Thyroid hormone inhibition	Improved	Serum	Cardiomyocytes	24, 25
Vitamin B supplementation	Improved	Systemic	Cardiomyocytes	31
Bmp2b overexpression	Improved	Ubiquitous	Cardiomyocytes	32
Noggin overexpression	Impaired	Ubiquitous	Cardiomyocytes	32
PRC2 sequestration	Impaired	Ubiquitous	Cardiomyocytes	35
Notch signalling upregulation	Improved	Endocardium	Endocardium	40, 41, 42
Trpv4 channel loss-of-function	Impaired	Endocardium	Endocardium	42
Klf2a loss-of-function	Impaired	Endocardium	Endocardium	42
dnVegfaa overexpression	Impaired	Ubiquitous	Endothelial cells	45
Neuropilin loss-of-function	Impaired	Epicardium	Endothelial and Epicardial cells	48
Osteopontin expression	Increases scar formation	Macrophages	Macrophages	49

Toll-like receptor signalling activation	Improved	Systemic	Macrophages	27
<i>tnfrsf9a upregulation</i>	Impaired	Endocardial cells	Macrophages	52
Foxp3a deficiency	Impaired	Regulatory T cells	Regulatory T cells	51