# An Evolving Role for Notch Signaling in Heart Regeneration of the Zebrafish *Danio rerio*

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Through the late twentieth and into the twenty-first century, the zebrafish *Danio rerio* has emerged as an invaluable model organism, affording scientific advancements and insights in a vast array of disciplines such as regenerative medicine, developmental biology, and stem cell research. While zebrafish have been used to model disease processes and to study the phenotypic consequences of genetic mutation, their capacity for regeneration of fin and heart have also received special attention in an accumulating body of biomedical literature. Given the rudimentary technology of the past, insight into the genetic programs and various components that may work to facilitate these regenerative processes remained obscure. It follows that an important first step in unraveling the zebrafish enigma was to investigate the fundamental question of genic changes that may occur in response to injury. From this understanding, further knowledge regarding the functional consequences of changes in gene expression, and ultimately the discovery of whole cellular pathways animating zebrafish regeneration, could then be used to deepen our understanding of this process in its various facets. The zebrafish has enjoyed wide regard in the annals of scientific literature, with exciting implications. This paper serves to follow the chronology of research, a mere twenty years as this work is current and ongoing, pertaining to the genetic programs underlying heart regeneration consequent to injury in the zebrafish (*Danio rerio)*.

With trendsetting research performed throughout the 1970’s and 1980’s, and the subsequent accumulation of great swaths of physiological, genetic, and developmental-biological data, the zebrafish was ready to make its debut as a model organism. At the turn of the new millennium, with a paucity of literature published regarding the mechanistic aspects of zebrafish heart regeneration and as interest swelled in biomedical academia, *Activation of Notch signaling pathway precedes heart regeneration in zebrafish* was published by Raya et al. in 2003. A mere

year previous, researchers demonstrated that if a segment of the zebrafish ventricle is excised, then complete regrowth of the tissue occurs. Raya et al. elaborated upon this work with their serendipitous discovery that various components of the Notch signaling pathway are upregulated in response to ventricular excision, implicating its role in facilitating cardiac regeneration. They also demonstrated that the regenerative process was genetically distinct from embryonic cardiac development (Raya et al., 2003). These results fundamentally altered the trajectory that this course of research would subsequently take. Transgenic adult zebrafish (CARP-EGFP) were anesthetized and underwent ventricular incision at the apex of the heart; transgenic embryos for myosin light-chain 2a (mlc2a)-EGFP underwent amputation of the posterior atrium at 24-hpf (hours post-fertilization). In addition, caudal fin amputation was performed in 6–9-month-old zebrafish, and the fish given 24-, 48- and 72-hours’ regeneration time. At the appropriate time, the caudal fin was removed from the anesthetized animal and prepared for fluorescence in-situ hybridization (FISH). Hearts were removed and fixed in a 4% paraformaldehyde (PFA) solution and prepared for cryosectioning as the ventricle was separated into 14mm segments and fixed to slides to be dried. In-situ hybridization was performed, and probes were obtained by RT-PCR or screening of cDNA in zebrafish libraries. Whole-mount hybridization was performed on zebrafish embryos and fins. A bromodeoxyuridine (BrdUrd) solution was injected into the abdominal cavities of anesthetized fish once daily for approximately one week. Following injection, fish were incubated in a BrdUrd solution. After a week, hearts were removed and prepared slide-fixed according to the aforementioned protocol. Slides were prepared for incubation with a murine primary BrdUrd antibody, then incubated in a secondary antibody, and slides were washed in polybutylene terephthalate and subsequently stained with diaminobenzidine and counterstained with eosin. FISH and RT-PCR analysis found that

reference genes involved in cardiac development were not upregulated in regenerating hearts, and that those involved in cardiac regeneration were not expressed in embryonic hearts. It was also found that notch1b was weakly expressed in control hearts but upregulated in regenerating hearts early after amputation (it remained upregulated for about 1 week after amputation and began to decline around 2 weeks) (Raya et al., 2003). DeltaC, a Notch ligand which colocalizes with Notch1b, was also upregulated in regenerating hearts and followed a similarly transient expression pattern post-amputation (Raya et al., 2003). Neither are expressed in developing hearts, reiterating that heart development and regeneration are distinct genetic programs.

Notch1b and DeltaC expression were not found in non-regenerating fins, but 24-hours post- amputation their expression could be visualized by FISH, and in close proximity with the msxB/C genes that encode homeodomain transcription factors important in cell-fate decisions (Raya et al., 2003). The most striking finding was that Notch pathway components are upregulated following msx upregulation in the early regeneration of both cardiac and caudal tissue. The evidence pointed to a role for the Notch pathway in mediating the regenerative response to injury in zebrafish hearts. The authors appropriately projected that these findings would have implications in regenerative medicine and stem cell biology (Raya et al., 2003). It was already known that the proliferation and differentiation of various stem cell types is Notch- dependent, and the authors emphasized that if these cell types exist in the zebrafish heart and play any part in the regenerative process was as of yet unknown.

Raya et al.’s groundbreaking research elucidated a convincing genetic mechanism underlying zebrafish heart regeneration and found a host of genes associated with the Notch cascade to be transiently upregulated following ventricular amputation. While the genetic architecture of the Notch pathway had already been implicated in the proliferation of various cell

types, Raya et al. poignantly noted that whether the cell fates of certain components of the heart were mediated by Notch signaling remained to be seen. Whether the Notch cascade was involved in reconstructing the cellular composition of the heart was a gap in our understanding to be filled, which Zhao and colleagues would address in 2014. Zhao et al. aptly stated that despite enormous biomedical relevance, “…the genetic and cellular determinants of natural cardiac regeneration remain incompletely characterized.” (Zhao et al., 2014). The article takes this body of research beyond simple correlates of temporal gene expression and onto the Notch-dependent proliferation of cardiomyocytes, an essential component of the myocardium and a cell type especially dense in the ventricle of the heart. Zhao et al.’s 2014 article found that suppression of Notch signaling greatly impaired cardiac regeneration and induced fibrotic scarring at the ventricular amputation site. Myocardial proliferation was also quantified and found to be significantly decreased, which the authors suggest reiterates Notch’s imperative role in cardiomyocyte proliferation. Overactivation of Notch also resulted in suppressed growth. Zhao et al.’s evidence points to a particular reactivity of cardiomyocyte proliferation to changes in Notch signaling during the regenerative process and demonstrates that a particular balance in Notch activity must be achieved for the proper repair of injured cardiac tissue.

In 2014 Zhao et al. published a paper entitled *Notch signaling regulates cardiomyocyte proliferation during zebrafish heart regeneration*, which sought to understand whether cardiac regeneration, specifically cardiomyocyte proliferation, was Notch-dependent. The researchers wanted to observe the regulation of Notch receptors following ventricular amputation and to

elucidate Notch’s role in mediating a response, particularly regarding endocardial and epicardial cell activation. A significant component of their efforts also involved surveying the phenotypic effects of Notch overexpression and under-expression. Expression patterns of zebrafish Notch

1a, 1b, 2, and 3 receptors (analogous to mammalian Notch 1, 2, 3, and 4) in uninjured hearts and injured hearts were analyzed at seven days post-amputation using in-situ hybridization. All four receptors were expressed in the endocardium, but none in the myocardium. This result was confirmed using fluorescent transgenic colocalization, and q-PCR verified these findings (Zhao et al., 2014). The Notch pathway was inhibited using the transgenic zebrafish strain Tg (hsp70: DN- MAML), MAML protein fused to GFP controlled by a heat shock promoter. This leaves Notch unable to commence, as truncated MAML proteins cannot incorporate the necessary cofactors. Ventricular apex amputation was performed on both controls and Tg (hsp70: DN- MAML) zebrafish, and cross-sections were analyzed for evidence of impaired regeneration.

After the sections were stained and analyzed, no differences between any three layers (epicardium, endocardium, myocardium) were immediately found. The Tg (hsp70: DN- MAML) zebrafish were then analyzed for cardiomyocyte dedifferentiation/proliferation defects post- injury. The transgenic line Tg (gata4: DsRed2), which expresses DsRed2 fluorescent protein in dedifferentiated myocytes, was utilized. Comparison of myocardial proliferation between control and Tg (hsp70: DN- MAML) zebrafish hearts at 7- and 14-days post-amputation used the myocardial marker Mef2 and the DNA replication marker Proliferating Cell Nuclear Antigen (PCNA). Control hearts displayed proliferative indices of 20-25%, while Tg (hsp70: DN- MAML) hearts had indices much lower, about 40-53% of control values at the same point in time (Zhao et al., 2014). This finding reveals that Notch signaling is imperative in cardiomyocyte proliferation post-injury. Temporal modulation of Notch was also found to be important in this process. Notch suppression during cardiomyocyte proliferation greatly decreased proliferative efficacy, while suppression at 1-2 days post-amputation did not significantly reduce proliferation (Zhao et al., 2014). This led the authors to conclude that Notch

signaling is required for, and coincides with, cardiomyocyte regeneration. Notch was hyperactivated using a GAL4/UAS transgenic line and heat-shocking induced activation post- injury. Cardiac amputation was performed on double transgenic Tg (hsp70: Gal4); Tg (UAS: NICD) zebrafish, of whom were then heat-shocked once daily for thirty days. Another transgenic line, Tg (hsp70: NICD), where notch activity is modulated by heat-shock and its subsequent impact on Notch Intracellular Domain (NICD) concentration, was also used. Myocardial regeneration was examined in cross-sections. Notch 1a, and to a lesser extent Notch1b were expressed in uninjured endocardium, while Notch 2 and 3 were strewn in low densities. Post- amputation, Notch1a, 1b, and 3 expressions were dramatically upregulated, with the highest expression proximal to the wound site (Zhao et al., 2014). Notch 1a and 2 were strongly upregulated in the epicardium covering the wound. Notch 3 was interestingly unaffected.

Myocardial cells appeared to have no Notch expression before or after injury, while injury- dependent upregulation was found in the endocardium and epicardium (Zhao et al., 2014). Heat- shocked Tg (hsp70: DN- MAML) zebrafish resulted in GFP-bearing embryos that appeared phenotypically similar to those whose Notch cascades had been compromised by mutation. Q- PCR found that two known Notch targets, Hey2 and Her4, were downregulated by heat-shocking Tg (hsp70: DN- MAML) fish. Mutations in these targets confer the same resultant phenotypes, making this strain a good inducible model for Notch inhibition (Zhao et al., 2014). Control animals exhibited robust regeneration, with new myocardial tissue at the apex. Transgenic zebrafish, by contrast, failed to regenerate, and showed fibrin and collagen scarring at the amputation site (this evidences that Notch suppression results in regenerative failure in amputated hearts). DsRed-positive cells were found in the myocardium, indicating that Notch is essential for cardiomyocyte proliferation (Zhao et al., 2014). Heat-shock of double transgenics

caused twice the stimulation of Hey2 and Her4 Notch target genes in embryogenesis—twice the level of endogenous Notch activation. This resulted in great myocardial deficits within the walls of the ventricle. A similar decrease in myocardial proliferation was found in the Tg (hsp70: NICD) line (Zhao et al., 2014). This led to an important conclusion, that both Notch-enhanced and suppressed hearts exhibited decreased cardiomyocyte proliferation. This implies a particular sensitivity to Notch signaling, and that a certain level of Notch activity must be achieved for proper cardiomyocyte proliferation in the regenerative process. The authors demonstrated that injured hearts express different Notch receptors and at different densities in a tissue-specific manner, and, perhaps most importantly, the phenomenon of injury-dependent Notch receptor upregulation generally. They also provided evidence that both suppression and overactivation of Notch signaling causes deficits in myocardial cell proliferation exclusively. Due to the lack of Notch activity in the myocardium, the authors propose a potential paracrine signaling mechanism (Zhao et al., 2014). Zhao et al. noted a desire for longer-term experiments analyzing tissue- specific Notch signaling in the layers of the heart, especially upon injury. They also poignantly called for further research into the downstream targets of Notch signaling that may facilitate cardiomyocyte proliferation and cardiac regeneration, an elaboration on that which was obscure in 2014 and remains so even now.

Zhao et al.’s work detailing the consequences of modulating Notch expression, as well as the elucidation of tissue-specific Notch receptors, naturally led to a deeper interest in the cellular components and ligands for the signaling cascades involved in regeneration. Having moved past Raya et al.’s rudimentary genic evidence, Zhao and his colleagues showed explicitly that Notch signaling was indeed activated in direct response to ventricular amputation. While both teams used similar procedures, one demonstrated the expression of genes associated with products of

the Notch pathway, while the other showed clear evidence for the pathway’s induction as a consequence of injury, in addition to its proliferative effect on myocardial cells. Between Raya et al. and Zhao et al. is a wonderful elaboration on a central theme—that Notch is activated post- injury and affords the zebrafish heart its regenerative capacity. Armed with the evidence of previous research, Li et al.’s 2021 paper detailing the mechanisms of activation in endocardial Notch signaling and its respective roles offers further clarification on the pathway’s involvement in regeneration and just how this may be mediated. In addition to offering a concise review of various Notch receptors and ligands, mechanisms of the pathway’s activation and its general function in development, etc., the authors give an exhaustive and contemporary review on Notch’s role in heart regeneration. Commenting on both Raya et al. and Zhao et al., the article illustrates a clear evolution on the state of knowledge regarding the myriad processes underlying zebrafish cardiac regeneration, and Notch’s role.

In addition to showcasing a rapidly evolving body of literature concerning zebrafish heart regeneration, Li et al.’s 2021 work recapitulates aforementioned themes like Notch’s role in cardiomyocyte proliferation and in cardiac regenerative processes overall. The authors focused on Notch activation subsequent to changes in hemodynamics following ventricular ablation, ventricular amputation, and cryoinjury in zebrafish larvae. In *The roles and activation of endocardial Notch signaling in heart regeneration*, Li and colleagues go beyond a simple reiteration of facts regarding the cellular and molecular functions of Notch, delving into the mechanosensitive cilia that respond to shear stress, force of hemodynamic alterations, and pressure overload following ventricular ablation, as well as the consequent *klf2* (Krüppel-like Factor 2) expression that may mediate the Notch response, cardiomyocyte proliferation, and ventricular remodeling. This research allowed the Notch pathway and its regenerative effects to

be surveyed at a finer scale than any previous work to-date had achieved. After reviewing nearly two decades of literature concerning Notch-mediated cardiac regeneration, particularly receptors, ligands, activation, and downstream targets (including the HER/HEY/HES gene family referenced by Zhao et al.), special focus was given to the most current research, concerning Notch activation in response to hemodynamic changes following ventricular injury (Li et al., 2021). This research is imperative in that it implicates the Notch cascade in multiple forms of ventricular injury in addition to just amputation—ablation, cryoinjury, and pressure overload, suggesting at present a common role for Notch in remedying cardiac injury. The function of MAML as a Notch coactivator was also revealed, illustrating why Zhao et al.’s heat-shocked Tg (hsp70: DN- MAML) transgenic fish exhibited decreased proliferative indices for cardiomyocytes (Li et al., 2021). With the advent of more precise technology, the authors were able to better uncover potential mechanisms underlying heart regeneration, showing Notch activation and subsequent myocardial Erbb2 and BMP signaling (both of which have been implicated in cardiomyocyte proliferation) post-ablation (Li et al., 2021). The mechanism for how Notch stimulates Erbb2 and BMP remains elusive, as does their respective roles in cardiomyocyte proliferation or the regenerative process. The consensus remains that the specific cellular/molecular aspects must be further studied (Li et al., 2021). Commentary on Zhao et al.’s follow-up study to their 2014 work was included, in which transcriptomic analysis showed that inhibition of endocardial Notch signaling caused reduced expression of Wnt antagonists *wif1* and *notumb1b*)—Wnt activity subsequently increased and exhibited the capacity to inhibit cardiomyocyte proliferation and cardiac regeneration. This deficit could be partly rescued with the Wnt inhibitor IWR (Li et al., 2021). Li et al. concluded that Notch signaling is activated by increased oscillatory flow in ablated hearts, mediated by mechanosensitive endocardial cilia.

*Klf2* was found to respond to hemodynamic changes, often through Notch, and expression of both *klf2a* and *klf2b* were increased in ablated hearts (Li et al., 2021). The colocalization of each in the ventricle implies their function in ventricular regeneration through Notch. In *Klf2a/2b* mutants, *klf2b* expression is increased in *klf2a* mutants and vice-versa, suggesting a compensatory mechanism (Li et al., 2021). Notch activation was decreased greatly in *klf2* mutants, and this correlated with reduced expression of cardiac transcription factors and decreased cardiomyocyte proliferation (Li et al., 2021). This may imply that hemodynamic changes induce Notch through *klf2*, and that both components are essential for cardiac regeneration. *Klf2* expression was downregulated in ift88 mutant ablated hearts, and Notch signaling was also greatly impaired and cardiomyocyte proliferation decreased. Ift proteins regulate cilia assemblage (Li et al., 2021). This result is particularly important, as it suggests a role for cilia in streamlining responses to hemodynamic changes and inducing cardiac regeneration. The mechanosensitive ion channel Trpv4 interacts with cilia to induce *klf2a* expression in valvulogenesis, and both *klf2a* and Notch expression are greatly reduced in Trpv4-/- knockouts (Li et al., 2021). The impaired expression of early cardiac transcription factors in utero, coupled with decreased cardiomyocyte proliferation and cell reprogramming, suggests that the Trpv4 channel may aid in *klf2*-Notch induction, and activation of the zebrafish cardiac regeneration program. While the molecular mechanisms remain to be explored, Li et al. concluded with some poignant questions regarding hemodynamic alterations and their potential role in damage and regenerative responses across species: How do cilia and mechanosensitive ion channels work together, and what is involved in this signal transduction? What are other ligands for Notch activation, and where can they be found? (Li et al., 2021). These are but the next questions to be addressed regarding zebrafish heart regeneration. Li et al. beautifully

reviewed the previous literature and thus-known components of the Notch pathway and went on to give the deepest analysis of Notch induction in response to cardiac injury to-date. This mechanistic, genetic, and molecular evidence will be an invaluable asset to the next group of researchers to happen upon the zebrafish cardiac regeneration hype.

In the rich tradition of scientific research, pushing the envelope and posing new questions can reverberate profoundly, until a new body of literature amasses into a novel framework by which pressing matters of future work can be addressed. Raya et al.’s initial research serendipitously revealed the upregulated expression of both Notch receptors and ligands during zebrafish heart regeneration and demonstrated that a distinct genetic network than that which is utilized during cardiogenesis in utero was deployed to mediate the regenerative process (Raya et al., 2003). This research perhaps singlehandedly established the zebrafish *Danio rerio* as a model for studying cardiac regeneration and predicted accurately the impact that subsequent research would have in advancing many fields on the cutting edge of modern science. Rightfully so, Raya et al.’s work set the tone for nearly the next two decades in regenerative-medical science, as labs around the world pursued the role of Notch signaling with equal fervor and at ever-finer scales.

Where Raya and colleagues left off, voicing the need for further research on Notch’s role in

cellular proliferation, Zhao et al.’s manuscript offered continuity, with its focus on tissue-specific Notch receptors and their upregulation in response to injury. In addition to establishing a direct link between Notch and cardiomyocyte proliferation, Zhao and his team were able to show the phenotypic implications of modulating Notch expression post-injury, thus elucidating functional aspects of the pathway in regeneration (Zhao et al., 2014). Nearly twenty years after Raya and colleagues’ pioneering work, Li et al. offer a contemporary perspective on Notch’s role in regeneration, enquiring into specific biomechanical cues that may mobilize the cascade. Their

perspective on the mechanosensitive cilia that respond to altered flow dynamics through the Trpv4 ion channel and consequent Notch-*klf2* coactivation offers the deepest explanation yet of how Notch may mediate regenerative change (Li et al., 2021). The finding that Notch activation was downregulated, and cardiomyocyte proliferation decreased in ift mutants, proteins regulating cilia assembly, further implicated their role in Notch signaling. Myocardial Erbb22 and BMP were activated upon Notch induction, both of which take part in cardiomyocyte proliferation.

Being the most contemporary research on these matters, the questions with which the authors conclude may represent the next wave in zebrafish cardiac regeneration research. What are the cellular and molecular mechanisms behind Erbb22 and BMP signaling in Notch activation and cardiomyocyte proliferation? How do mechanosensitive cilia and Trpv4 ion channels work in concert to sense hemodynamic changes post-injury, and how is signal transduction mediated? Before this area of research can move forward, these pressing questions must be tended to. From Raya et al.’s simple genic evidence, Li et al. has likely homed in on the very particulars facilitating Notch activation in response to injury, with their hemodynamic response model and its various functional components and downstream targets. The compelling conclusions gleaned from this work could have tremendous implications for the future of biomedical and translational research. But for this to happen, matters of basic science must be addressed first; the cellular and molecular devices regulating zebrafish heart regeneration need be understood in great detail before therapeutic strategies can be devised. For all its promise, this compelling body of literature remains incomplete and in its relative infancy.

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