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REVIEW

The heart of the neural crest: cardiac neural crest cells in development and regeneration

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ABSTRACT

Cardiac neural crest cells (cNCCs) are a migratory cell population that stem from the cranial portion of the neural tube. They undergo epithelial-to-mesenchymal transition and migrate through the developing embryo to give rise to portions of the outflow tract, the valves and the arteries of the heart. Recent lineage-tracing experiments in chick and zebrafish embryos have shown that cNCCs can also give rise to mature cardiomyocytes. These cNCC-derived cardiomyocytes appear to be required for the successful repair and regeneration of injured zebrafish hearts. In addition, recent work examining the response to cardiac injury in the mammalian heart has suggested that cNCC-derived cardiomyocytes are involved in the repair/regeneration mechanism. However, the molecular signature of the adult cardiomyocytes involved in this repair is unclear. In this Review, we examine the origin, migration and fates of cNCCs. We also review the contribution of cNCCs to mature cardiomyocytes in fish, chick and mice, as well as their role in the regeneration of the adult heart.

KEY WORDS: Cardiac neural crest cells, Cardiac specification, Myocardium, Heart development, Cardiac regeneration

Introduction

Neural crest cells (NCCs) are a multipotential population of cells unique to vertebrates that arise after delamination from the dorsal-most aspect of the neural tube, migrate ventrally along the anterior-posterior axis, and contribute to a diverse number of tissues (Trainor, 2014). Cardiac neural crest cells (cNCCs) are a subpopulation of NCCs that give rise to specific structures within the heart. In birds and mammals, these cells originate between the otic placode and the 3rd somite, and they migrate into the caudal pharyngeal arches (PAs; the 3rd, 4th and 6th PAs). They eventually give rise to a multitude of structures (Fig. 1A) including: the pharyngeal arch arteries, which undergo remodeling to give rise to the aorta and pulmonary trunk; the cap of the intraventricular septum (IVS), the developing outflow tract (OFT) cushions, which differentiate into the aortic and pulmonary valves; and the parasympathetic innervation of the heart (Kirby and Waldo, 1990, 1995; Farrell et al., 1999; Bronner, 2012; Etchevers et al., 2019; Yamagishi, 2020).

In recent years, there has been a growing body of evidence to suggest that, in addition to their established roles in cardiovascular development, cNCCs in small numbers can contribute to the formation of cardiomyocytes, and that these cNCC-derived cardiomyocytes may play an important role in cardiac regeneration. In this Review, we focus on recent developments in understanding cNCC biology and discuss publications that report a cNCC contribution to cardiomyocytes and heart regeneration.

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The origin, migration and cell fate specification of cNCCs

cNCCs were first identified in quail-chick chimera and ablation experiments as a subpopulation of cells that contribute to the developing aorticopulmonary septum (Kirby et al., 1983). cNCCs are induced by a network of signaling factors such as BMPs, FGFs, NOTCH and WNT in the surrounding ectoderm that initiate expression of cNCC specification genes (Sauka-Spengler and Bronner-Fraser, 2008; Scholl and Kirby, 2009). Transcription factor networks that include *Msx1* and *Msx2*, *Dlx3* and *Dlx5*, and *Pax3* and *Pax7* are also associated with NCC induction (Kwang et al., 2002; Robledo et al., 2002; Goulding et al., 1991; Basch et al., 2006) (Table 1).

Between embryonic day (E) 8.5 and 9.0 in the developing mouse, cNCC specification genes including *FoxD3*, *Snai1* and *AP-2* (*Tfap2a*) are expressed in the dorsal neural tube (Dottori et al., 2001; Kos et al., 2001; Murray and Gridley, 2006a; Schorle et al., 1996) (Table 1). Other important cNCC specification genes are the SRY-related HMG-box family of genes, *Sox9* and *Sox10* (Aoki et al., 2003; Cheung and Briscoe, 2003; Tani-Matsuhana et al., 2018). SOX9 primes pre-migratory cNCCs for epithelial-to-mesenchymal transition (EMT) partly by activating the *Snai2* (also known as *Slug*) promoter (Sakai et al., 2006), whereas *Sox10* expression is maintained within cNCCs during specification and migration (He and Soriano, 2015). A number of *Sox10* lineage-tracing tools have therefore been developed in fish and mouse models to mark the cNCC lineage and examine its contribution to tissues (discussed in detail below, see also Table 2) (Matsuoka et al., 2005; He and Soriano, 2015).

Once induced, cNCCs delaminate from the neural tube, while undergoing EMT, and emigrate into the OFT as well as into the proximal portions of the PA arteries. There are various models for how cNCCs migrate through the PAs. These include: (1) the ‘contact inhibition of locomotion’ model, in which two NCCs collide and change direction (Roycroft and Mayor, 2016); (2) the ‘trailblazer cell’ model, in which gene expression changes in the leading cells induce the movement of follower cells (McLennan et al., 2015); (3) the ‘chemoattractant and repellent’ model, in which the signaling molecules from surrounding tissues guide migrating cNCCs (Hutson et al., 2006); and (4) the ‘co-attraction model’, in which the cNCCs express factors that maintain cNCCs together via chemotaxis during migration (Carmona-Fontaine et al., 2011). Multiple gene pathways have been implicated in these processes driving migration of cNCCs (see Table 1).

During delamination from the neural tube and migration, cNCCs are mesenchymal in terms of their morphology, develop filipodia (long cytoplasmic projections that interact with the surrounding extracellular matrix) and express the intermediate filament protein

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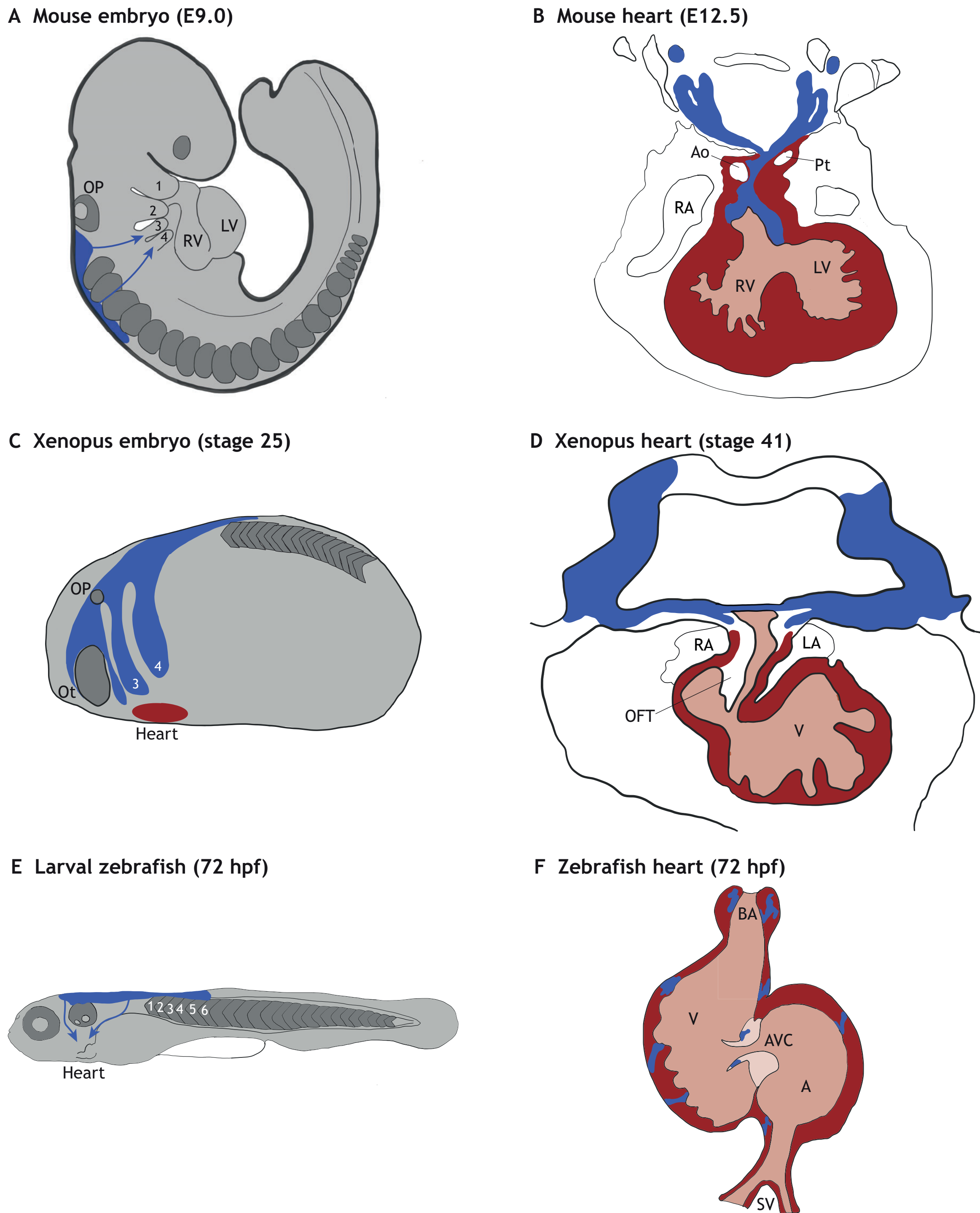

Fig. 1. cNCC contributions to the developing heart. (A) E9.0 mouse embryo (21 somite stage). cNCCs (blue) arising between the otic placode and the 4th somite migrate in waves through the 3rd and 4th pharyngeal arch arteries (labeled 3 and 4 in figure) into the heart through the developing outflow tract (not shown). (B) E12.5 day embryonic heart showing cNCCs that remodel and form the septation between the aorta and pulmonary trunk, and the membranous septum between the right and left ventricles. Myocardium and cardiac lumen are indicated in red and light red, respectively. (C) Tailbud stage *Xenopus laevis* embryo, stage 25. Lateral view showing cNCCs in the 3rd and 4th migratory stream populating the heart mesoderm (red). (D) Cross section of stage 41 *Xenopus laevis* embryo showing cNCCs that remain confined to the aortic sac and arch arteries and do not enter the outflow tract cushions. (E) Larval zebrafish 72 hpf. cNCCs arising at the level of the otic vesicle to the 6th somite are shown migrating through the embryo to populate the developing heart through the arterial and venous pole. (F) Cross section of 72 hpf zebrafish heart showing cNCCs that migrate into the developing segments of the zebrafish heart, including the bulbus arteriosus, ventricle, AV junction and atrium. These represent 12-20% of the cells in the zebrafish heart at this stage. A, atrium; Ao, aorta; AVC, atrioventricular junction; BA, bulbus arteriosus; LA, left atrium; LV, left ventricle; OFT, outflow tract; OP, otic placode; Ot, optic vesicle; Pt, pulmonary trunk; RA, right atrium; RV, right ventricle; SV, sinus venosus; V, ventricle.

vimentin (Kirby and Hutson, 2010). In mice, *Snai1* expression marks pre-migratory and post-delamination cNCCs, whereas *Snai2* expression marks only migrating cNCCs (Murray and Gridley, 2006a). Interestingly, SNAI1 and SNAI2 are not necessary for cNCC migration, as cNCCs in double-mutant mice can migrate (Murray and Gridley, 2006b); instead, SNAI1 and SNAI2 appear to

Table 1. Genes/gene families involved in cNCC development: summary of mouse and non-mammalian experimental results.

Roles	Gene/family name	Experimental results in mouse model	Experimental results in non-mammalian models
Induction from non-neural ectoderm and paraxial mesoderm	*Wnt*	*Wnt* signals are thought to be important for the formation of NCC (Bhatt et al., 2013)	*Wnt* signals (i.e. *Wnt1*) are required for NCC induction in *Xenopus* (Guémar et al., 2007)
	RARs	Lack of RARs in NCC does not affect NCC specification in mouse embryos (Dupé and Pellerin, 2009)	In *Xenopus* and quails, RARs are required for proper NCC induction (Villanueva et al., 2002)
	Fgf8	*In vivo* reporter assays demonstrate that *Fgf8* is not expressed in premigratory or migratory NCCs (Frank et al., 2002)	Contrasting results have been observed in zebrafish and *Xenopus* (Roehl and Nüsslein-Volhard, 2001, Villanueva et al., 2002)
	Notch	*Notch* signaling pathway is active in NCCs; however, it is not essential for NCC induction (Mead and Yutzey, 2012)	Notch signaling is required for NCC induction in frog and chick (Glavic et al., 2004, Endo et al., 2002)
Neural plate border specifiers	*Pax7*	*Pax7* expression at E7.5 at the tips of the lateral neural folds marks NCC (Murdoch et al., 2012)	In chick embryos, *Pax7* is one of the earliest markers of NCC (Basch et al., 2006)
	Pax3	*Pax3* expression in the dorsal region of the neural groove at E8.5 marks where NCCs are specified (Epstein et al., 2000)	A reduction of *Pax3* expression affects NCC induction in *Xenopus* (Monsoro-Burq et al., 2005)
	Dlx5	*Dlx5* expression at E7.25 at the edge of the neural folds in the mouse embryo marks NCC (Yang et al., 1998)	In *Xenopus* and chick, *Dlx5* is expressed at the borders of the neural plate (McLarren et al., 2003)
	Zic	*Zic5*-deficient mice exhibit impaired neural tube closure and facial skeletal abnormalities (Inoue et al., 2004)	Loss-of-function studies suggest that *Zic1* regulates NCC specification genes in *Xenopus* (Sato et al., 2005)
	Msx1/*Msx2*	*Msx1*$^{-/-}$;*Msx2*$^{-/-}$ mouse embryos exhibit neural tube closure defects (Ishii et al., 2005)	In *Xenopus*, *Msx1* is important for expression of NCC gene markers (Tribulo et al., 2003)
	AP-2	*AP-2* null mouse embryos display impaired closure of the cranial portion of the neural tube at E13.5 (Schorle et al., 1996)	*AP-2* is required for the specification of a subpopulation of NCC in zebrafish, and required for NCC induction in *Xenopus* (Meulemans and Bronner-Fraser, 2002, Knight et al., 2003, Luo et al., 2003)
Specification	*Sox8*	*Sox8*-deficient mice do not exhibit developmental defects at either embryonic or postnatal stages (Sock et al., 2001)	*Sox8* plays an essential role in the activation of expression of NCC markers in *Xenopus* (O'Donnell et al., 2006)
	Sox9	*Sox9* can be detected in the dorsal region of the neural tube at E9.5, from which NCCs emerge. *Sox9* null mouse embryos exhibit late differentiation effects (Mori-Akiyama et al., 2003)	*Sox9* is important for NCC development in zebrafish and *Xenopus* (Yan et al., 2005, Spokony et al., 2002)
	Sox10	*Sox10* cells are observed emerging from the dorsal region of neural tube at E8.5 (Britsch et al., 2001)	*Sox10* transcripts in chick can be detected dorsally in the neural tube at HH stage 9 (Cheng et al., 2000)
	FoxD3	*FoxD3* can be detected in the dorsal region of the neural tube at E9.5, from which NCCs emerge (Dottori et al., 2001)	*FoxD3* misexpression leads to upregulation of migratory NCC marker genes in chick embryos (Dottori et al., 2001)
	Snail	*Snai1* can be detected dorsally in the neural folds. Knock-out of *Snai1* does not show impaired NCC formation (Murray and Gridley, 2006a)	Both *Snai1* and *Snai2* play a role in NCC induction in *Xenopus* (Shi et al., 2011)
Migration	*FoxD3*	*FoxD3*-positive migrating NCCs can be detected in the ventral and dorsolateral migration pathway (Dottori et al., 2001)	*FoxD3* controls different aspects of NCC migration in chicks (Dottori et al., 2001)
	Sox10	*Sox10* can detected in premigratory and migratory NCCs at E8.5 and E10.5, respectively (Britsch et al., 2001)	*Sox10* can be detected in the early and migrating NCC in chick (Cheng et al., 2000)
	Dvl2	*Dvl2* null mouse embryos exhibit cardiac structural defects owing to NCC abnormalities (Hamblet et al., 2002)	Inhibition of Dishevelled signaling impairs NCC migration in zebrafish (Banerjee et al., 2011)
	Cdh2 (*N-cadherin*)	*Cdh2* is expressed in migratory cNCCs (Luo et al., 2006)	*N-cadherin* inhibition results in impaired NCC migration in *Xenopus* (Theveneau et al., 2010)
	Vangl1/*Vangl2*	*Vangl1;Vangl2* double-mutant mouse embryos do not exhibit an NCC migration phenotype (Pryor et al., 2014)	A migration phenotype is evident in *Xenopus* and zebrafish (Matthews et al., 2008, Carmona-Fontaine et al., 2008)
Post-migration (heart)	*Cdh2*	*Cdh2* is required for proper development of OFT, as *Cdh2-cko* using Wnt1-Cre exhibit OFT structural abnormalities (Luo et al., 2006)	No data in non-mammalian systems
	Hand1/*Hand2*	Both transcription factors can be detected in NCCs and NCC-derived tissues (Srivastava et al., 1997, Firulli et al., 1998). *Hand2 Wnt1Cre* CKO exhibit OFT defects (Firulli et al., 1998, Morikawa and Cserjesi, 2008, Holler et al., 2010)	In chick, *Hand1*/*Hand2* can also be detected in NCC-derived tissues (Angelo et al., 2000)
	Foxc1/*Foxc2*	Expressed in cardiac NCC and NCC-derived cardiac tissues. Mice null for both genes exhibit OFT defects (Seo and Kume, 2006)	A role for the ortholog *foxc1a* in cardiac pathologies shown in zebrafish (Yue et al., 2018)
	Mef2c	*Mef2c* is detected in the NCC within the branchial arches at E9.0 (Lin et al., 1998)	In zebrafish, *mef2ca* detected in the branchial arches from 24 hpf to 32 hpf (Miller et al., 2007)
	Semaphorins	*Sema3C* transcripts can be detected in the OFT at E10.5 (Feiner et al., 2001)	*Sema3D* is expressed in the OFT of chick (Bao and Jin, 2006)

control precise EMT events at the neural plate border by modulating expression of the cadherin family of transmembrane proteins (Taneyhill and Schiffmacher, 2017). Pre-EMT cNCCs also express N- and E-cadherin; however, migratory cNCCs exclusively express E-cadherin (Dady et al., 2012; Scarpa et al., 2015). It is also well-established that the secreted extracellular signaling molecule WNT1 plays an essential role in the induction and emigration of cNCCs from the neural tube (Dorsky et al., 1998; Fenby et al., 2008). *Wnt1* is expressed within early migrating cNCCs and is rapidly downregulated as cells emigrate ventrally towards their final destinations (Burstyn-Cohen et al., 2004). As such, *Wnt1* transcriptional regulatory sequences have also been used to drive Cre recombinase, allowing NCC-specific recombination of reporter alleles knocked into the *Rosa26* locus and thus the tracing of cNCC lineage (Table 2) (Jiang et al., 2000). Other NCC-specific transgenic *Cre* drivers, such as *P0Cre* and *Pax3Cre* (Yamauchi et al., 1999;

Table 2. Transgenic lines used to identify cNCC contribution to cardiomyocytes

Allele	Description of allele	Species	Marks cardiomyocytes	Description of cNCC contribution to cardiomyocytes	Reference
Tg(-4.9sox10:egfp)ba2	Transgenic eGFP expression driven by NCC-specific −4.9 kb *sox10* promoter sequence	Fish	Yes		Carney et al., 2006
Tg(cmlc:GFP-sox10: ERT2-Cre)	*Cardiac myosin light chain* (*cmlc*)-expressing cardiomyocytes marked by GFP. Tamoxifen-inducible *cre* line driven by −4.9 kb *sox10* promoter sequence	Fish	Yes	Volume of *sox10*-derived cardiomyocytes averages 0.07% in adult zebrafish hearts; 14 days post injury, *sox10*-derived cardiomyocytes are 6%	Mongera et al., 2013, Sande-Melon et al., 2019
Tg(-5sox10:GAL4,UAS: Cre)	*Cre* recombinase expression driven by the NCC-specific −4.9 kb *sox10* promoter sequence	Fish	Yes	*Sox10* lineage NCC-derived cardiomyocytes account for 12% of cells in heart at 30 hpf	Cavanaugh et al., 2015
Tg (sox10:Cre;cryaa: dsRed)	*Cre* recombinase expression driven by NCC-lineage specific *sox10* −7.2 kb promoter sequence	Fish	Yes	NCC-derived cardiomyocytes contribute to 12% of cardiomyocytes in the heart at 48 dpf	Abdul-Wajid et al., 2018
Sox10-MCS4-c-Fos-Cre/ Sox10MCS4Cre	*Sox10* neural crest enhancer (MCS4) driving *Cre* expression	Mouse	No		Stine et al., 2009
Tg(Sox10-icre/ ERT2)1Ldim/ Sox10ERTCre	Transgenic mouse with tamoxifen-inducible *Cre* in the *Sox10* locus	Mouse	No		Simon et al., 2012
Tg(Sox10-ERT2/cre/ ERT2)17Sor/ Sox10MCS4Cre2	*Sox10* MCS4 enhancer driving tamoxifen-inducible *Cre*	Mouse	No		He and Soriano, 2015
Tg(Pax3-cre)1Joe	*Cre* expression driven by 1.6 kb promoter of *Pax3*	Mouse	No		Epstein et al., 2000
Tfap2a$^{tm1(cre)Moon}$	Knock-in *IRESCre* cassette into *AP2α* locus at 3′ end between stop codon and endogenous poly A signal.	Mouse	No		Macatee et al., 2003
B6.Cg-Tg(Wnt1-cre)11Rth Tg(Wnt1-GAL4) 11Rth/J (in text *Wnt1Cre*)	Cre insertion between a 1.3 kb 5′ *Wnt1* promoter and coding sequence, including 5.5 kb 3′ UTR	Mouse	Yes	*Wnt1Cre* lineage cardiomyocytes by eGFP labeling 17% of trabeculated myocardium in the proximal half of the ventricle at E15.5	Jiang et al., 2000, Tang et al., 2019
Wnt1-Cre2((129S4-Tg(Wnt1- cre)1Sor/J)) (in text *Wnt1Cre2)*	Transgenic mouse with 5′ 1.3 kb *Wnt1* promoter, *Cre* gene and 5.5 kb *Wnt1* 3′ UTR	Mouse	Yes	Similar cellular contributions compared with *Wnt1Cre* (Jiang et al., 2000) observed	Lewis et al., 2013, Tang et al., 2019
Tg(Wnt1-cre/Esr1)10Rth* (in text *Wnt1ERTCre*)	Tamoxifen-inducible transgenic *Cre* driven by 5′ 1.3 kb *Wnt1* promoter and 5.5 kb *Wnt1* 3′ UTR	Mouse	Yes	NCC-derived cardiomyocytes detected by GFP reporter labeling in the left ventricle of 10-week-old mice. No cell-type quantification was performed	Danielian et al., 1998, Tamura et al., 2011
Tg(P0-Cre)94Imeg/ P0-Cre	Cre recombinase expression driven by 1.1 kb promoter of *protein 0* (*MPZ*)	Mouse	Yes	NCC-derived lineage labeling 0.2% cardiomyocytes by eGFP reporter at 1 day old	Yamauchi et al., 1999; Nakamura et al., 2006
Kit$^{tm1(cre/ERT2)Dsa}$	Knock-in tamoxifen-inducible *Cre* inserted into the ATG start codon in exon 1 of the *Kit* locus	Mouse	Yes	*cKit*-lineage by eGFP reporter labeling 30% of atrial and ventricular cardiomyocytes at E18.5	Hatzistergos et al., 2015

Epstein et al., 2000), have also been generated (see Table 2), although many of these present with advantages as well as caveats (see Box 1).

Once the PA arteries are populated by cNCCs, the arches undergo remodeling. cNCC-derived cells contribute to the vascular smooth muscle of the dorsal aorta, the brachiocephalic root of the internal carotid, and the pulmonary arteries (Fig. 1B,C) (Bronner and Simões-Costa, 2016). Post migration, the streams of cNCCs coalesce at their destination tissue and either directly contribute to the final tissue or initiate their final cell fate differentiation processes (Kirby and Hutson, 2010). Signaling between cNCCs and their associated endocardial/myocardial tissue is required for proper tissue morphogenesis. Indeed, defects in these processes have been linked to multiple diseases and are associated with congenital heart defects (Hutson and Kirby, 2007; Keyte and Hutson, 2012).

The cell fate of cNCCs appears to be established early. For example, there is clearly some level of cell fate specification in NCCs that is conveyed by their location (i.e. anterior-posterior); whereas cranial NCCs normally contribute to the bone and cartilage of the face, transplanted trunk NCCs are unable to contribute to the facial structures in chick (Simoes-Costa and Bronner, 2016). Further work has shown that the forced expression of *Sox8*, *Tgif1* and *Ets1* within trunk NCCs in chick embryos can reprogram these NCCs into cNCCs (Gandhi et al., 2020). There is also evidence to suggest that cNCCs make cell fate decisions post-migration. For example, TWIST1 loss-of-function analysis in mice shows that OFT cNCCs adopt a neuronal phenotype, forming ganglia-like structures within the OFT cushions (Vincentz et al., 2008, 2013). In addition, the use of an NCC-*Cre* (*Hand1^{eGFPCre}*) for *Twist1* deletion post-migration shows that the OFT NCCs still adopt a neuronal cell fate, thus demonstrating that these cells have the capacity to respond to their local environment as well as receiving fate instructions from their origin. More recent single cell spatial transcriptomics in mice reveal that TWIST1 overexpression in pre-EMT stage NCCs can transform trunk NCCs to a more cranial NCC phenotype (Soldatov et al., 2019). Taken together, these data suggest that the modulation of key

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Box 1. The caveats of lineage tracing cNCCs

Although a powerful tool, the use of *Cre*-recombinase approaches to lineage map cells can be misleading. *Cre* lineage tracing with cNCC markers such as *Sox10* and *Wnt1* had suggested some contribution from cNCC lineage to cardiomyocytes; however, this was not observed using other cNCC-lineage markers, indicating some amount of variability in the type of cNCCs that can give rise to cardiomyocytes. Also, *Cre* lineage tracing tools can exhibit problematic ectopic recombination that would be followed by misinterpretation of cell fates observed in adult tissues. *Cre* lineage tracing after myocardial injury also has its caveats; for example *de novo* activation of the *sox10-cre* driver could result from the injury itself and not actually reflect an NCC-origin. Inducible *cre* lines, such as the zebrafish *GFP-sox10:ERT2-Cre*, which require multiple tamoxifen treatments to induce recombination, could also be a source of unintended CRE activity. *Cre*-lines employing *sox10* enhancers are indeed a powerful tool, but the disconnect between *cre* expression and *sox10* mRNA expression that has been reported must be considered seriously when attributing parental lineage. Clearly using multiple cell markers has been established as a requirement to make rigorous cell lineage calls; however, if a *sox10cre* allele can be upregulated in an acute responsive way (for example, after injury), the lineage conclusions drawn would be in error. Do we need to employ multiple lineage tools (*Cre* and *Dre* for example) to double-label NCC to account for this possibility? Would a *Wnt1-Dre*; *Sox10-Cre*-labeled cell be found in the heart at similar percentages as the current body of work suggests? Such caveats are difficult to resolve until further investigation is employed to address these discrepancies.

gene regulatory networks governing cNCC fate specification can alter cNCC fate. Further dissection of these networks could identify new genes that can be used to further our understanding of the cNCC lineage.

cNCC contributions to the myocardium: species-specific variations?

Insights from classical studies in zebrafish

The first reports of cNCCs contributing to the cardiomyogenic-lineage came from studies performed in zebrafish (*Danio rerio*) embryos. The two-chambered zebrafish heart is morphologically and functionally distinct from the four-chambered mammalian heart (Fig. 1). For example, although both zebrafish and mammalian hearts exhibit uni-directional flow during embryonic stages, the adult mammalian heart functions under far higher pressure load to maintain separate oxygenated and deoxygenated circulation circuits. In contrast, zebrafish maintain a uni-directional circulation system that pushes blood through the vessels in the gills allowing diffusion of oxygen into the blood, which is then pushed through the entire body. In addition, the majority of mature cardiomyocytes in the mouse heart are binucleated and are recalcitrant to cell division. By contrast, cardiomyocytes in zebrafish are smaller, mono-nucleated and, through a number of elegant studies, have been shown to retain proliferative abilities (Fig. 2A) (Poss et al., 2002; Jopling et al., 2010; Kikuchi et al., 2010).

A cNCC contribution to cardiomyocytes in the zebrafish heart was first suggested by Sato and Yost, who employed Alexa Fluor 488-labeled dextran NCCs transplanted into unlabeled age-matched host embryos (Sato and Yost, 2003). They used immunohistochemistry with the MF20 monoclonal antibody, which is specific for cardiac αMyosin heavy chain (González-Sánchez and Bader, 1984), to validate that the cNCCs had differentiated into cardiomyocytes. These experiments demonstrated that NCC-derived cardiomyocytes are present within the myocardial layers of the bulbus arteriosus and ventricles (Sato and Yost, 2003). In another study by Kirby and colleagues, cNCC lineage tracing was performed using laser irradiation to uncage fluorescein from DNMB-caged fluorescein dextran in zebrafish (Li et al., 2003). Cells with activated fluorescein dye were then tracked for lineage, and cNCC locational inferences were verified histologically and molecularly using *in-situ* hybridization for *tcfap2a* and *tcfap2b*, which marks pre-migratory NCCs, and immunohistochemistry with anti-HNK1 antibodies, which label migrating cNCCs (Li et al., 2003). Laser ablation of cNCC in these embryos resulted in decreased cardiac function as measured by ejection fraction and stroke volume (Li et al., 2003). Together, these studies suggested that, in zebrafish, cNCCs contribute to the myocardium during development.

Transgenic zebrafish lines

The classical approaches discussed above have limitations, including dilution of the dye with each cell division, accessibility challenges for direct injection, cell fusion events leading to mislabeling of lineages, dye from dead cells being picked up by surrounding living cells, and positional accuracy variation for laser ablation experiments. To address these limitations, more recent studies have used transgenic lines to determine cNCC contributions to the developing zebrafish heart. Early experiments tracing the cNCC lineage in zebrafish employed a −4.9 kb *sox10* promoter sequence upstream of the translation start site to drive *egfp* expression (Table 1) (Carney et al., 2006). Multiple laboratories have utilized this −4.9 kb *sox10* promoter element to drive reporter or *cre* expression within zebrafish cNCCs. Using this approach, it was reported that −4.9 kb *sox10*-lineage marked cells adopt a myocardial fate in the uninjured zebrafish heart as well as after injury (Mongera et al., 2013; Cavanaugh et al., 2015; Sande-Melón et al., 2019; Tang et al., 2019). Chen and colleagues combined the −4.9 kb *sox10-cre* with a floxed-reporter for an *mCherry*-nitroreductase fusion protein (Cavanaugh et al., 2015). When treated with metronidazole, cells expressing the *mCherry*-nitroreductase fusion protein undergo cell death via enzymatic conversion of metronidazole to a toxic compound that cell-autonomously kills the nitroreductase-expressing cell (Pisharath and Parsons, 2009). This approach thereby allows for both cell fate analysis (via epifluorescence of the *mCherry* allele) and targeted ablation of *sox10*-derived NCCs. These studies revealed that within the developing zebrafish heart, ∼12% of sox*10* lineage marked NCCs are observed at 30 h post fertilization (hpf) and 48 hpf. There is also an observed decrease in the overall percentage of *sox10* lineage cells at later stages of development, likely the result of additional second heart field (SHF) cardiac progenitors being added from the arterial and venous poles of the heart. At 48 hpf, the *sox10* lineage cells have become α-actinin-expressing cardiomyocytes and their localization appears more restricted to the base of the ventricle, the AV boundary and the proximal atrium (Cavanaugh et al., 2015). More recently, Yost and colleagues employed refined genetic tools to permanently mark individual zebrafish cells with a dual transgenic *cre* system that labels both *sox10*-expressing cells (with RFP) and cells that express the cardiomyocyte-specific protein Myosin light chain 7 (Myl7; with GFP) (Abdul-Wajid et al., 2018). In these experiments, transgenic *cre* expression is driven by a larger −7.2 kb version of the *sox10* promoter element that includes an additional 2.3 kb upstream of the *sox10* promoter sequence (Table 2). Both the −4.9 kb and −7.2 kb *sox10* promoter fragments drive similar expression patterns within NCCs (Hoffman et al., 2007). In the adult zebrafish, 12% of the total number of cardiomyocytes at 48 hpf are RFP+, indicating that they are *sox10* lineage-derived cardiomyocytes; this increases to 15% at 72 hpf (Abdul-Wajid et al., 2018). The ablation of *sox10* lineage cNCCs at 48 hpf using metronidazole treatment leads to a loss of the Notch ligand *jag2b* within cNCC-derived cardiomyocytes. This

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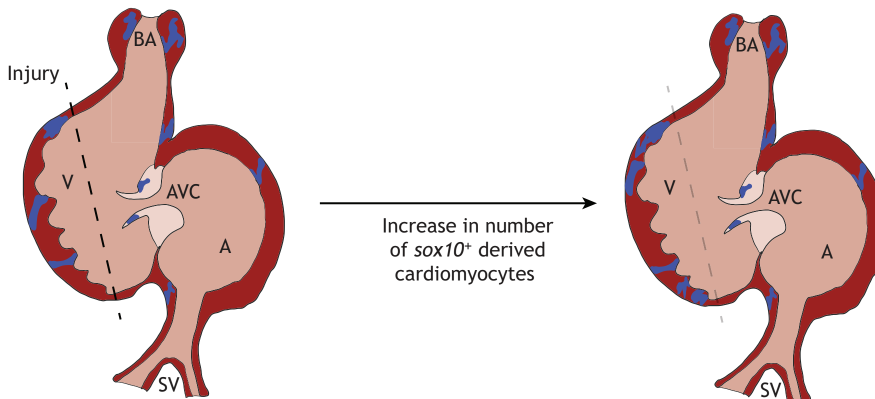

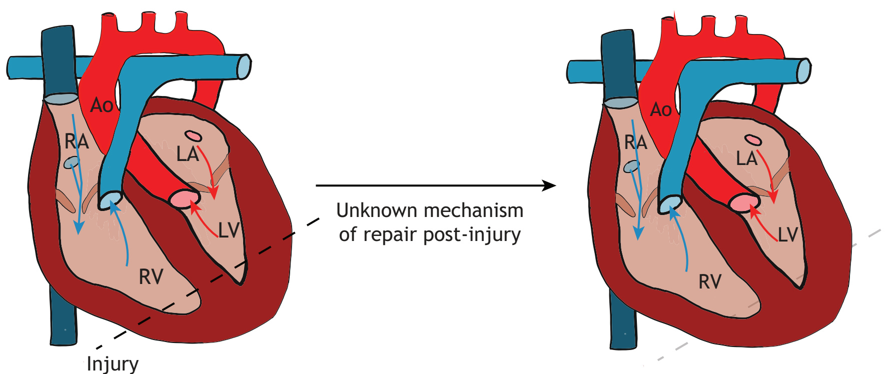

Fig. 2. cNCCs in heart regeneration. (A) Injury to the ventricle (dashed line) in an adult zebrafish heart leads to an increase in the number of *sox10*+ cardiomyocytes (blue). Specifically, these *sox10*+ cardiomyocytes are seen close to the site of injury. Myocardium and lumen are in red and light red, respectively. (B) Injury to the ventricle in the postnatal mouse heart from P0 to P3 results in successful repair. The contribution of cNCCs or *Sox10*+ cells to cardiac repair in the mouse heart is still unknown. A, atrium; Ao, aorta; AVC, atrioventricular junction; BA, bulbus arteriosus; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; SV, sinus venosus; V, ventricle.

decrease in *jag2b* is proposed to account for the observed aberrant ventricular trabeculation and adult hypertrophic cardiomyopathy, leading the authors of this study to suggest that cNCC-derived cardiomyocytes act as a source of Notch signaling required for patterning trabeculation during heart development (Abdul-Wajid et al., 2018).

Studies in *Xenopus*

cNCCs have also been characterized in the frog *Xenopus laevis,* which possesses a three-chambered heart, utilizing *sox10* as a marker to follow migrating cNCCs during cardiac morphogenesis (Lee and Saint-Jeannet, 2011; Alkobtawi et al., 2018) (Fig. 1). Unlike other vertebrate groups such as fish, birds and mammals, amphibians contain cNCCs that are confined to the aortic sac and PAs; amphibian cNCCs do not enter the OFT cushion. NCCs from *X. laevis* embryos expressing the reporter RFP were transplanted into an unlabeled host to lineage trace these cells, revealing an NCC contribution to cardiac development (Lee and Saint-Jeannet, 2011). However, co-labeling of the cardiomyocyte-specific marker MF20 excluded a contribution of cNCCs to cardiomyocytes, indicating that cNCCs in *X. laevis* do not give rise to cardiomyocytes (Lee and Saint-Jeannet, 2011). Thus, the transition from a common ventricular chamber found in the amphibian heart to a fully divided systemic and pulmonary circulatory system in warm-blooded vertebrates might have been made possible by the ingress of cNCCs into the OFT septum, enabling a true separation of oxygenated and deoxygenated circulations.

Studies in mice

The potential contribution of cNCCs to cardiomyocytes has also been examined in mice. Analysis of the NCC lineage using various *Cre* alleles, including *Wnt1-Cre* (Jiang et al., 2000), *P0-Cre* (Yamauchi et al., 1999), *Pax3-Cre* (Epstein et al., 2000) and *AP-2αCre* (Macatee et al., 2003), suggests that mature cardiomyocytes could have a contribution from the cNCC lineage although, as discussed below, these lineage-tracing tools have their own caveats (Box 1; Table 2). Work by Fukuda and colleagues suggested that stem cells of an NCC origin, marked by *P0-Cre* (Yamauchi et al., 1999), are present within the ventricular myocardium and can contribute to the replacement of cardiomyocytes post-injury (Table 2) (Tomita et al., 2005; Tamura et al., 2011). However, subsequent work using a dual recombinase reporter labeling system reported that only pre-existing myocytes give rise to the observed replacement of cardiomyocytes, as determined by examining >6000 sections at 12-h intervals from 12 h to 28 days post injury, effectively ruling out all non-cardiomyocyte lineages as a potential ‘stem cell’ source post injury (Li et al., 2018).

These results have recently been called into question as a growing number of studies employing newer mouse models suggest that murine cNCCs may harbor the ability to differentiate into cardiomyocytes. A study in mice using an NCC-specific *Sox10* enhancer located −28.5 kb upstream of the transcriptional start site to drive Cre Recombinase (Table 2) reported that marked cells are present within the cardiac OFT at E15.5 (Stine et al., 2009). Another study generated a tamoxifen-inducible transgenic *iCre* using a *Sox10* BAC clone to insert the *Cre* cassette into exon 3, which includes the SOX10 initiation codon (Table 2) (Simon et al., 2012). Induction of the Cre recombinase activity by administering tamoxifen also revealed *Sox10*-lineage cells within the heart; however, neither the location nor the identity of the *Sox10+* cells was defined (Simon et al., 2012). An important caveat to both these

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studies is that co-labeling with a cardiomyocyte-specific marker was not demonstrated; therefore, the cell fate reported to be marked by *Sox10-Cre* in these experiments cannot definitively be identified as cardiomyocytes.

The contribution of cNCCs to cardiomyocytes within the mouse heart has also been analyzed using labeling with the stem cell marker *c-Kit*, which is expressed within the early neural tube at E9.0 and has been shown to contribute to endothelial/endocardial cells of the OFT, atria and ventricles (Wilson et al., 2004; Hatzistergos et al., 2015; Sultana et al., 2015). Surprisingly, using both inducible and non-inducible *c-Kit-Cre* mice, results show that *c-Kit* lineage cells contribute to only a small percentage (0.005%) of cardiomyocytes *in vivo*, and much lower than the known physiological rate of turnover of cardiomyocytes (van Berlo et al., 2014; Sultana et al., 2015). These data support the conclusion that a significant and functional contribution of *c-Kit* lineage cells to the heart is unlikely (van Berlo et al., 2014; Sultana et al., 2015). In addition, work by Bin Zhou and colleagues used multiple recombinases and reporter lines to first label all cardiomyocytes within the heart and then examine the lineage of new cells that arise post-injury (He et al., 2017; Li et al., 2018). Using different inducible-*Cre* lines marking endocardial cells, mesenchymal stromal cells, resident and activated fibroblasts, endothelial cells, pericytes, smooth muscle cells, as well as epicardial cells, the authors tested the contribution of these differentiated cell lineages to adult cardiomyocytes, and none were found to produce cardiomyocytes within the mouse adult heart (Li et al., 2018).

Another report demonstrated co-labeling of *Wnt1Cre* lineage cells (Jiang et al., 2000) with TBX3-expressing cardiomyocytes within the IVS (Miquerol et al., 2013). However, the *Wnt1Cre* transgenic mouse employed in this work contains an in-frame start codon arising from vector sequence splicing (Lewis et al., 2013), resulting in overexpression of the *Wnt1* transcript by as much as 67-fold, as determined by qRT-PCR in the E14.5 embryonic head. This overexpression results in increased proliferation, potentially contributing to any observed phenotypes (Lewis et al., 2013). To address this caveat, a new *Wnt1Cre2* transgenic mouse was developed by using the same 1.3 kb 5′ promoter sequence in combination with the 5.5 kb 3′ region but without the *Wnt1* gene coding sequence to drive *Cre* expression (Table 1) (Lewis et al., 2013). By combining this transgenic mouse with an *R26mTmG* reporter to lineage mark NCCs, Bronner and colleagues examined the contribution of cNCCs to the myocardial lineage during mouse development (Tang et al., 2019). They demonstrated that *Wnt1Cre2* lineage NCCs expressing GFP from the *R26mTmG* floxed allele also exhibited co-immunostaining with the cardiomyocyte-specific marker troponin-t; these cells were present within the OFT, IVS and ventricular myocardium of mouse hearts at E15.5 as well as postnatally [postnatal day (P) 2] within these structures. Their analysis also revealed that 17% of trabeculated ventricular myocardium appears to be NCC-lineage cardiomyocytes, a number similar to that observed in the *sox10*-lineage analysis in fish (see above) (Fig. 1E) (Tang et al., 2019). However, there are reports of ectopic labeling using the *Wnt1Cre2*, and more refined genetic tools are required to accurately lineage map cNCCs in mice (see Box 1).

New data from chick

Although the contribution of cNCCs to the myocardium had been reported in mammals and fish, there was no direct evidence to support similar contributions in birds. However, recent work from the Bronner lab has examined whether the NCC-lineage contributes to cardiomyocytes in avian hearts (Tang et al., 2019). In these experiments, an H2B-YFP-labeled replication-incompetent avian (RIA) virus was injected into the hindbrain lumen of chick embryos between Hamilton Hamburger (HH) stages 9-10 to transduce-mark pre-migratory NCCs. The subsequent analysis of embryos at HH20 revealed that labeled cells were detected within the myocardium of the OFT and ventricles (Tang et al., 2019). Similar to the mouse *Wnt1Cre2* lineage-traced cells, RIA-labeled avian cNCC-derived cardiomyocytes did not undergo active cell proliferation or apoptosis, indicating that cNCC-lineage-cardiomyocytes remain stable over time, both post-migration and post-differentiation. This direct-labeling and lineage-tracing approach is the first demonstration of NCC contribution to cardiomyocytes in the chick embryo, establishing an important evolutionary data point and supporting the hypothesis that cNCCs are capable of entering the cardiomyocyte lineage.

cNCC contributions to heart innervation and the cardiac conduction system

The sympathetic and parasympathetic innervation of the heart is derived from cNCCs that give rise to largely catecholaminergic and acetylcholinergic neurons and the supporting cells of the cardiac ganglia (Kirby et al., 1983; Howard and Bronner-Fraser, 1985). Direct contribution of cNCCs to essential components of the cardiac conduction system has also been reported by Nakamura et al., who used a tamoxifen-inducible *Wnt1* 5′ 1.3 kb promoter and 5.5 kb 3′ untranslated region (UTR) driving *Cre* expression (*Wnt1ERTCre;* Table 2) (Danielian et al., 1998; Nakamura et al., 2006). The examination of hearts at E17.5 revealed *Wnt1ERT* expression in the proximal cardiac conduction system, including the posterior internodal tract, His bundle and bundle branches (Nakamura et al., 2006).

cNCC contributions to the cardiac conduction system have also been investigated in chick embryos. For example, it was demonstrated that laser-induced ablation of cNCCs leads to ectopic epicardial breakthroughs as well as apex-to-base propagation defects, suggesting a functional role for cNCC-derived cardiomyocytes in patent cardiac conduction (Gurjarpadhye et al., 2007). In contrast, experiments by Kelly and colleagues employed *Connexin40-GFP* (*Cx-40*) along with *Wnt1Cre* (Jiang et al., 2000) and *R26LacZ* to co-label the ventricular conduction system (Table 2) (Miquerol et al., 2013). This study reported no observed co-labeling of conduction system cells with GFP and β-galactosidase activity, suggesting that cNCC lineage cell contributions may have been misattributed to the conduction system fate without the use of a secondary marker for confirmation (Miquerol et al., 2013; Mohan et al., 2018).

The role of cNCC-derived cardiomyocytes in cardiac regeneration

Understanding variations in the repair process (i.e. the robust regeneration seen in some fish and amphibians compared with the ineffective repair observed in birds and mammal species) has been a topic of intense investigation for many years (Oberpriller and Oberpriller, 1974; Flink, 2002; Hsieh et al., 2007; Laflamme and Murry, 2011; Tzahor and Poss, 2017; Cardoso et al., 2020) (Fig. 2). In this section, we examine the molecular mechanisms by which regeneration could be taking place and we review work carried out in various species examining whether cNCC-derived cardiomyocytes can contribute to heart regeneration.

Cardiac regeneration in *Xenopus* is an interesting undertaking, and understanding such species-to-species variations observed in cardiac regenerative capacities may provide clues to crucial mechanisms.

Mechanical amputations of 10-15% of the ventricular apex in *X. laevis* tadpoles lead to complete heart regeneration (Marshall et al., 2019). The replacement cardiomyocytes responsible for this regeneration are identified as existing cardiomyocytes (as observed in fish) that, through a thyroid hormone-dependent mechanism, re-enter the cell cycle post injury, proliferate and re-differentiate to functional tissue (Marshall et al., 2019). In contrast, similar ventricular resections at the apex of juvenile *X. laevis* hearts (i.e. post-metamorphosis) reveal that cardiomyocytes lose regenerative capability within the resected hearts (Marshall et al., 2019). Interestingly, the regenerative capability of the post-metamorphosis heart within anuran amphibians appears variable. Studies performed on the closely related *X. tropicalis* reveal that pre-existing cardiomyocytes in this species are fully capable of efficacious adult cardiac regeneration (Liao et al., 2017). An attractive hypothesis offered to help explain this variation between these two frog species is the variation in the levels of thyroid hormones observed post injury. Indeed, cardiac regeneration in *X. laevis* is impaired when the thyroid hormone triiodothyronine (T3) is in excess or blocked, suggesting that T3 levels must be precisely regulated (Marshall et al., 2019). However, precisely how thyroid hormone levels differ in *X. tropicalis* is currently unknown. In mice, there is also an experimental association with thyroid hormone levels and cardiac regeneration (Hirose et al., 2019) and gaining a better understanding of the mechanism by which thyroid hormone levels modulate cardiomyocyte proliferation will be important for understanding the species-specific variations observed in heart regeneration.

Studies in the early 2000s showed that adult zebrafish hearts can fully regenerate after removal of up to 20% of total ventricular mass (Poss et al., 2002). The source of the cells contributing to this regenerative capacity are clearly pre-existing mature cardiomyocytes (Jopling et al., 2010; González-Rosa et al., 2017). However, as we discuss below, work from multiple groups has revealed that cNCC-derived cardiomyocytes may be a significant source of replacement cardiomyocytes in zebrafish.

In one study, Cavanaugh et al. employed metronidazole treatment of zebrafish embryos between 4 hpf and 48 hpf to ablate *sox10*-lineage marked cNCCs via *mCherry*-nitroreductase to induce cardiac repair. This study also used co-labeling via Myl7-GFP to specifically mark cardiomyocytes. The results revealed aberrant cardiomyocyte cell morphology as well as a decrease in heart rate. A significantly smaller ventricular chamber is also observed, although this is reported to be caused by a reduction in SHF cell recruitment to the heart. These findings could suggest a role for cNCC-derived cardiomyocytes in zebrafish cardiac regeneration; however, the ablation of cNCCs that would normally differentiate into cardiomyocytes (i.e. OFT-generating cNCCs) could affect SHF-derived cardiomyocytes, thus also contributing to the observed phenotype (Cavanaugh et al., 2015). Work from the Bronner lab revealed that, when 20% of the ventricular apex of the adult zebrafish is surgically removed, *sox10+* lineage cardiomyocytes (identified via the −4.9 kb *sox10Cre* promoter transgene driving an eGFP reporter; Carney et al., 2006) are observed 7 days post injury at the injury border zone and spread throughout the ventricle by 21 days (Tang et al., 2019). Using the −4.9 kb *sox10*:*cre:mCherry* transgenic zebrafish (Cavanaugh et al., 2015), this study went on to show that *mCherry+* cNCC-derived cardiomyocytes are present within the compacted and trabeculated layers of regenerated ventricular myocardium (Tang et al., 2019). Moreover, RNA-seq performed on *sox10+* FACS-sorted cardiomyocytes 21 days post injury revealed that, in addition to being more transcriptionally active, the *sox10+* lineage cells exhibit gene regulatory network signatures resembling those observed in NCCs during embryonic stages (Tang et al., 2019). However, the authors did not rule out the possibility that the cells reactivating the *sox10*/NCC program post injury may arise from another cell lineage that is capable of *sox10* upregulation; this is a major caveat to any *Cre* lineage mapping data (Box 1).

To refine whether embryonically-derived *sox10+* lineage NCCs or adult *sox10*-expressing cardiomyocytes contribute to zebrafish heart regeneration post injury, Mercader and colleagues used the −4.9 kb *sox10* promoter element that drives tamoxifen-inducible *cre* (Mongera et al., 2013; Sande-Melón et al., 2019). Adult *sox10*-expressing cardiomyocytes were labeled by hydroxytamoxifen treatment overnight. In the adults, 0.07% of the volume of *mCherry+* cells relative to all *MHC+* (*myh6+*) cells (i.e. all cardiomyocytes) are *sox10+* in uninjured hearts, and this increases to 5% at 14 days post injury. This is in contrast to previous studies that used an embryonically active −4.9 kb sox10 lineage *cre* (Cavanaugh et al., 2015; Abdul-Wajid et al., 2018), which reported that 12% of cardiomyocytes in adults were derived from the *sox10+* lineage. These adult *sox10* lineage-derived cardiomyocytes are capable of becoming transcriptionally active compared with cardiomyocytes not having expressed *sox10*. Furthermore, gene and pathway enrichment analyses revealed that adult *sox10* lineage-derived cardiomyocytes respond to injury with an increase in cell proliferation and cell motility (Sande-Melón et al., 2019). Ablation of *sox10+* cardiomyocytes in adult zebrafish before cardiac injury (using either *sox10*-derived cell-specific diphtheria toxin expression or metronidazole treatment) results in increased fibrotic tissue deposition but without a significant reduction in animal survival or cardiac function. These data suggest that *sox10*-marked NCC-derived cardiomyocytes are necessary for effective zebrafish heart regeneration. Tamoxifen administration at 12-48 hpf timepoints also allowed labeling of embryonic *sox10*-derived cNCC cardiomyocytes. Lineage tracing of these embryonic *sox10*-derived cNCC cardiomyocytes showed no contribution during repair after cryoinjury (Sande-Melón et al., 2019). However, one serious caveat to these observations is the inconsistency between the *sox10-cre*-mediated GFP expression and the endogenous *sox10* mRNA expression (Sande-Melón et al., 2019). This could indicate that lineage tracing with the −4.9 kb promoter *sox10 cre* allele alone is not sufficiently rigorous to pinpoint a cNCC-derived cardiomyocyte. It is also unknown whether a similar loss of regenerative capacity is seen when non-*sox10*-expressing cardiomyocytes are ablated. A direct functional correlation between the embryonic NCC-lineage and adult heart regeneration has therefore still not been confirmed. Furthermore, the contribution of other cell types, such as neuronal- or non-cNCC mesodermal lineage cells, to cardiac regenerative potential remains untested.

In a recent study, Stainier and colleagues interrogated changes in chromatin accessibility in zebrafish hearts that have undergone cryoinjury by using a transgenic line that marks all *gata4*-expressing cardiomyocytes with a reporter (*gata4:egfp+*) to find transcription factor regulators that are important for heart regeneration (Beisaw et al., 2020). They reported that the transcription factor activator protein (AP-1) is necessary for zebrafish heart regeneration and cardiomyocyte proliferation. They further reported that AP-1 contributes directly to chromatin remodeling at genes that are involved in sarcomere disassembly and cardiomyocyte protrusion, a process by which cytoskeletal rearrangement allows cytoplasmic projections into the extracellular matrix, normally seen in migrating cells and also in cardiomyocytes that undergo mitosis (Ridley, 2011; Morikawa et al., 2015). It would be interesting to analyze whether the AP-1-positive cells of this study also express *sox10*.

Similar to the observations made between *Xenopus* species, there is also variability in the cardiac regenerative response within

teleosts; medaka (*Oryzias latipes*), unlike its cousin the zebrafish, does not exhibit a cardiac regenerative response to injury (Ito et al., 2014). The variation observed between these two fish has been attributed to variations in their immune responses to injuries (Lai et al., 2017). Interestingly, recent work from the Molkentin lab shows that, indeed, non-cellular activation of the innate immune response is sufficient to induce cardiomyocyte repair following injury in mice (Vagnozzi et al., 2020). This repair response is linked to macrophage activation, which leads to changes in cardiac fibroblast activity and reduces the extracellular matrix content at the site of injury (Vagnozzi et al., 2020). How this acute immune response is linked to cardiac repair is still largely unknown and continued investigations into its role in cardiomyocyte cell cycle reentry should prove informative.

In the mouse, cardiac regeneration post-injury is limited; cardiomyocyte proliferation rates are ∼1% per year in mice and humans (Soonpaa and Field, 1997; Pasumarthi et al., 2005; Bergmann et al., 2009; Soonpaa et al., 2013; Lázár et al., 2017). Moreover, work from multiple labs has revealed that removal of up to 15% of the cardiac ventricles can trigger a robust regenerative response in neonatal mice (Porrello et al., 2011; Morikawa et al., 2015; Soonpaa et al., 1996). However this repair response is limited by P2 and lost by P3, after which the hearts become recalcitrant to cardiomyocyte replacement (Notari et al., 2018). The major impediment to murine cardiac regeneration appears to be the inability of cardiomyocytes to become proliferative (Pasumarthi and Field, 2002). Gaining an understanding of the molecular mechanisms driving cardiomyocyte cell cycle re-entry has been rigorously researched for the last two decades, with multiple pathways implicated in this process (Cardoso et al., 2020). To date however, based on results generated from current cell lineage tracing tools, it still remains unclear whether mouse cNCCs can adopt a cardiomyocyte lineage, let alone whether preexisting cNCC-derived cardiomyocytes can contribute to efficacious heart regeneration.

Conclusions

The neural crest is a fascinating and essential tissue, contributing to numerous tissues that align along the anterior-posterior axis in the developing vertebrate embryo. NCC-derived cardiomyocytes are likely the most controversial lineage attributed to NCCs. Nevertheless, the preponderance of zebrafish data suggests that cardiomyocytes are a *bona-fide* NCC end-point. The data showing that NCC-derived cardiomyocytes play key roles in zebrafish cardiac regeneration are exciting. They also raise the question of whether the lack (or significantly lower numbers) of these crucial NCC-derived cardiomyocytes in some fish, amphibians and all warm-blooded vertebrate cousins is a large part of the explanation as to why the damaged adult mammalian heart does not recover effectively over time. In addition, the significance of variations in the spatial distribution of cNCCs contributing to myocardium in amphibians or zebrafish, compared with birds and mammals, remains unclear and needs further exploration. The 3-day window during which the neonatal mouse heart remains capable of regeneration raises the question of whether or not this window corresponds to the cardiomyocyte expression of *Sox10* or another NCC gene regulatory network. In addition, it would be interesting to look further into the recent work on the differences in cardiac regeneration between cold and warm-blooded vertebrates, which have focused on the expression of thyroid hormone and suggest that NCC-derived cardiomyocytes could be sensitive to differences in thyroid hormone regulation (Hirose et al., 2019). Hypoxia-induced gene expression could also have ties to NCC migration and cardiomyocyte proliferation during regeneration (Barriga et al., 2013; Nakada et al., 2017), and it has been suggested that hypoxia induces regeneration in zebrafish by inducing cardiomyocyte dedifferentiation and proliferation (Jopling et al., 2012), although how this translates to mammalian systems is still unclear. Lastly, the immune response could be a major player in the process of allowing cardiomyocytes to re-enter the cell cycle. However, the question of whether cNCC-derived cardiomyocytes are targets of the immune response cannot be answered at this time.

Studies of early postnatal mouse hearts clearly show that proliferating cardiomyocytes are key to the regeneration of the adult mammalian heart after injury. Whether cardiomyocytes are capable of efficiently making this switch from a quiescent functional myocyte to a proliferative and reparative one also remains an open question. In addition to a change in cell cycle status, it would be interesting to determine whether the reparative cells originate from an NCC-derived and/or other lineages. What is currently clear is that, in all species in which cardiac regeneration is observable and efficacious to restoring function, the cell-source of the repair post myocardial injury is preexisting quiescent cardiomyocytes that are induced to re-enter the cell cycle. Are these cells cNCC-derived cardiomyocytes? Or are they a mix of cell lineages? These are currently important unanswered questions.

Further study of the gene regulatory networks involved in the repair mechanisms of the neonatal mammalian heart is also required, for example to determine whether the *Sox10*- or *Wnt1*-marked cNCC lineages contribute to this process. Would a *Sox10*-lineage derived population be amenable to cell cycle re-entry? Although not discussed in this Review, work examining the Hippo pathway has highlighted that this pathway is clearly important for the induction of a cardiac regenerative response in mammals. For example, work from the Martin lab demonstrates that the Hippo pathway is a key modulator of cell cycle re-entry for cardiomyocytes (Liu and Martin, 2019). Are cNCC derived cardiomyocytes more amenable to modulation from Hippo signaling? This will need to be addressed experimentally.

Many more questions lie ahead: would part of the cardiac repair response require the right concentrations of thyroid hormone, the right immune response or the right chromatin context? We have no definitive answers to these questions thus far, but it is clear that gaining a better understanding of the molecular mechanisms involved in cNCC induction, migration and cardiomyocyte specification is likely to contribute to the development of innovative approaches to treat cardiac injury.

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