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REGENERATIVE MEDICINE (SM WU, SECTION EDITOR)

Molecular Regulation of Cardiac Conduction System Development

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Abstract

Purpose of review The cardiac conduction system, composed of pacemaker cells and conducting cardiomyocytes, orchestrates the propagation of electrical activity to synchronize heartbeats. The conduction system plays a crucial role in the development of cardiac arrhythmias. In the embryo, the cells of the conduction system derive from the same cardiac progenitors as the contractile cardiomyocytes and and the key question is how this choice is made during development.

Recent Findings This review focuses on recent advances in developmental biology using the mouse as animal model to better understand the cellular origin and molecular regulations that control morphogenesis of the cardiac conduction system, including the latest findings in single-cell transcriptomics.

Summary The conducting cell fate is acquired during development starting with pacemaking activity and last with the formation of a complex fast-conducting network. Cardiac conduction system morphogenesis is controlled by complex transcriptional and gene regulatory networks that differ in the components of the cardiac conduction system.

Keywords Cardiac Conduction · Embryonic Development · Molecular Control · Morphogenesis · Transcription Factors

Introduction

The heart, along with the digestive system, is the only organ capable of initiating its own contractions. Cardiac automaticity is made possible by the presence of a natural pacemaker within the heart, the Sinoatrial Node (SAN) [1], which spontaneously generate action potentials (AP). In mammals and birds, the AP is then transmitted to the rest of the heart by a complex network of specialized cardiomyocytes (CMs) forming the cardiac conduction system (CCS) [2]. The various components of the CCS sequentially propagate the electrical impulse throughout the heart, ensuring the coordination and stereotypy of the beats (Fig. 1).

First, rapid propagation of the AP within the atria ensures synchronous contraction of both atria. The AP then reaches the unique electrical connection between the chambers: the atrioventricular node (AVN), where it is advantageously slowed, optimizing ventricular filling prior to contraction. The AP is then transmitted to the fast-conducting ventricular conduction system (VCS) or His-Purkinje. It passes through the His bundle or atrioventricular bundle (AVB), which then divides into the right and left bundle branches (RBB and LBB) on either side of the septum. The isolated RBB and LBB carry the AP along the interventricular septum to the apex and to the terminal part of the CCS: the Purkinje fiber (PF) network of each ventricle. Finally, the PF network conducts the AP along the ventricular free walls as it transmits the depolarization to the working myocardium, initiating ventricular systole from apex to base.

The electrophysiological characteristics of cardiomyocytes within the CCS result from the expression of a wide range of conduction-specific genes, including ion channels responsible for pacemaker activity and action potential characteristics, gap junctions responsible for conduction velocity, and low levels of contractile proteins [3–6]. Importantly, each compartment of the CCS is unique, with specific physiological and histological characteristics adapted to their role in cardiac conduction [7]. For example, both the SAN and the AVN rely on Hcn4, the main cation channel responsible for the I_{funny} current, which underlies their automaticity [8, 9]. Furthermore, the heterogeneous conduction velocities observed in the different compartments of the CCS are made possible by the expression of a combination of gap junctions with different conductance. Specifically, the SAN and AVN, characterized

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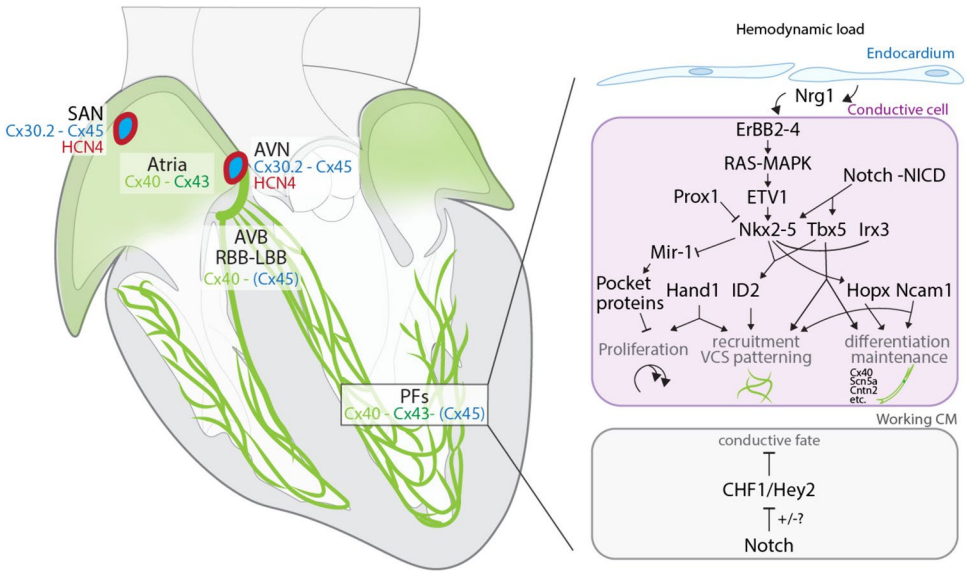

Fig. 1 The anatomy, conduction properties and molecular regulation of the development of the CCS. On the left, the anatomy and conduction properties of the different components of the murine or human CCS. The conduction velocity within the heart depends on the specific expression of connexins in different components of the CCS. On the right, interactions of known factors controlling the development of the VCS through the regulation of proliferation, recruitment and differentiation of conductive cells. SAN, sinus node; AVN, Atrioventricular node; AVB, Atrioventricular bundle; RBB, Right bundle branch; LBB, Left bundle branch; PFs, Purkinje fibers

by slower conduction, predominantly express connexins of low conductance, such as Cx45 and Cx30.2 (9pS) [3, 10]. In contrast, fast-conducting components like the atria, AVB, RRB LBB, LPF and RPF express Cx40, which forms high conductance gap junctions (~ 180pS) [3, 11] (Fig. 1).

Although CCS cells share characteristics with neurons, such as genetic markers, electrophysiological properties and electrical function [12], they are CMs. Indeed, retrospective clonal analysis, revealed a common cellular origin between conducting and contractile CMs [13, 14]. More precisely, the different compartments of the CCS show close lineage relationship with contractile cardiomyocytes from neighboring myocardium, suggesting that conductive and contractile properties are acquired during cardiac morphogenesis [15–18]. Thus, although all CCS compartments are integrated into a functional conducting pathway in the mature heart, their origin and developmental regulation largely differ.

Importantly, despite its small volume within the heart, the CCS plays a critical role in cardiac function and in the occurrence of cardiac arrhythmias [14, 19]. Understanding the precise regulation of CCS patterning during heart development is therefore of paramount importance. Due to the considerable influence of genetics, the mouse—which has a very similar morphology, function and molecular regulation of the CCS to that of humans [20, 21] – has become the animal model of choice for understanding the development of the CCS.

Genetic Control of SAN Development

The heart is the first functional organ in the embryo with transient calcium releases [22] and focal contractions starting as early as cardiac crescent stage [23, 24], and soon being replaced by peristaltic contractions. However, these first cardiac activities can initiate anywhere in the heart tube. A dominant pacemaker emerges at the inflow pole of the heart only after, in a region progressively enriched in Hcn4, which prefigures the emplacement of the SAN [25].

In the adult heart, SAN cells are characterized by the expression of a unique set of ion channels, i.e. Hcn4 and gap junctions, such as Cx45 and Cx30.2, which differs from the atrial myocardium that expresses Cx40, Cx43 and Nppa [26]. The SAN is divided into a large head and a tail forming a comma-shaped structure at the junction of the right atrium and superior vena cava [1]. Both parts of the SAN arise from a mesodermal pool of progenitors that diverges early from both the first and second heart fields (FHF and SHF) and that will also give rise to the sinus venosus (SV) [27–30]. The subdivision of the SAN is delineated by distinct gene programs with the expression of transcription factors *Tbx18*$^{+}$*/Nkx2-5*$^{-}$ in the head region and *Nkx2-5*$^{+}$*/Tbx18*$^{-}$ in the tail [30, 31]. Strengthening a bipartite model for SAN development, genetic tracing analyses using inducible *Hcn4-CreERT2* mice lines indicate a progressive temporal activation of Hcn4 in the SAN, with early expression in progenitors from the tail (from

E7.5) and later expression (from E8.5 onward) in the head [16].

The differentiation and maintenance of the pacemaker activity of the SAN is controlled by a gene regulatory network involving Tbx18, Tbx2, Shox2, Nkx2-5 and Isl1 [32, 33].

Illustrating the importance of Tbx18 for SAN development, inactivation of Tbx18 causes a hypoplastic SAN with absence of the head and reduced tail region [30].

At early stage of development, Short stature homeobox 2 (Shox2) gene expression is extended in the sinus venosus to cells presenting pacemaker activity and co-expressing Hcn4 and Nkx2-5 [34]. Later during development, pacemaker cells are restricted to the mature SAN under the control of a transcriptional balance between Shox2 and Nkx2-5 to regulate cell fate. Shox2 is not directly required for the pacemaker activity of the SAN but the conditional deletion of *Shox2* in *Nkx2-5* tail domain induces sick sinus syndrome in association with a loss of the junction between SAN and atria [34]. The T-box transcription factor Tbx3 is expressed in the developing conduction system and, similar to Shox2, acts as a transcription repressor of the working myocardium program by down regulating the expression of *Gja5, Gja1* and *Scn5A* in pacemaker cells [35]. Conditional overexpression of *Tbx3* converts atrial cells to a pacemaker phenotype characterized by a downregulation of *Cx43* and upregulation of *Hcn4* expression, which results in ectopic pacemakers [36]. SAN homeostasis in the adult is also tightly dependent on *Tbx3* expression indicating its importance not only for pacemaker specification but also for maintenance of conduction system integrity [37]. In contrast to these transcriptional repressors, *Isl1* encodes for a LIM-homeodomain transcription factor and serves as a positive transactivator for the expression of pacemaker specific genes like *Tbx3, Shox2* and *Hcn4* [38]. The conditional inactivation of *Isl1* specifically in the SAN at different stages of development reveals that Isl1 affects the SAN size and induces bradycardia by playing a cell-autonomously role in proliferation and differentiation of pacemaker cells [26, 39]. Moreover, any disturbances of *Nkx2-5* expression in the adult heart correlate with sinus diseases, showing that the regulation of this transcriptional cascade is also crucial for the maintenance of pacemaker phenotype [40]. Nkx2-5 is required to form the junction between tail and head. Indeed, inactivation of *Nkx2-5* in the *Shox2* domain does not hamper the development of the node but disturbs the atrial activation suggesting a precise role for this junction [41]. In summary, pacemaker cells originate from an independent mesodermal lineage that give rise to the sinus venosus and starts to synchronize heartbeats at E8.0. Defects in SAN development or dysfunction of pacemaker cells in either SAN compartment leads to bradycardia or sick sinus syndrome [42].

Genetic Control of AVN Development

The AVN is critical in slowing atrioventricular conduction, thereby introducing a necessary delay between atrial and ventricular contraction [43]. The AV delay functionally develops around E10, when the chamber-myocardium acquire a conductive phenotype [44–46], while the atrioventricular canal (AVC) and OFT, deprived of Cx40, remains slow [11]. As development progresses, the differentiation of the AVN is marked by the expression of Tbx3, Hcn4, Cx30.2 and Cx45, giving the AVN its pacemaker and slow conducting properties [10, 43, 47, 48]. In parallel, the reinforcement of electrical insulation through the development of fibrous tissues further isolates the AVN, leaving it as the only electrical communication between the atria and ventricles.

Genetic tracing using respectively *Tbx2* or *Hcn4* has shown that the AVN arises from the AVC [49, 50] and commit to a AVN fate by E10-E11 [18]. On the other hand, the annulus fibrosus arises from epithelial-mesenchymal transition (EMT) of epicardial cells at the AV junction [27].

The establishment of the AV junction relies on different signaling pathway on the right and left side. On the left side, both the transcriptional program of the AVC myocardium and establishment of the annulus fibrosus relies on Tbx2 [50]. Consequently, deletion of *Tbx2* results in accessory pathways, detectable anatomically and functionally by optical mapping [51]. On the other side, the establishment of the right AV junction depends on Notch and Wnt signaling [52, 53]. Accessory pathways are detectable after Notch activation or in a Wnt loss of function (LOF) model while Wnt activation induces ectopic annulus fibrosus in ventricular myocardium [52]. Rescue experiments suggest that Notch-mediated ventricular pre-excitation results in part from downregulation of Wnt signaling [54]. Notch inhibition also regulates the AV conduction pathway by interfering with AVN maturation. Indeed, downregulation of Notch signaling leads to formation of a small AVN associated with an elevated conduction velocity between atria and ventricles [53]. However, Notch signaling does not affect the early development of the AVC or the expression of Tbx3.

The specification and development of nodal cells is very sensitive to Nkx2-5 and Tbx3 gene dosage during embryonic development [55–58]. Indeed, AVN progenitors are absent in *Nkx2-5* null embryos, and a small AVN develops in *Nkx2-5* haploinsufficient mice or after postnatal deletion of *Nkx2-5* [56, 59]; likewise, *Tbx3* hypomorphic or AVC-deleted alleles cause AV blocks and hypoplasia of the CS. Moreover, inducible deletion of *Tbx3* in the adult heart causes AV blocks associated with a reduced number of AVN cells [37]. Interestingly, the severity of these AV blocks decreases after

a few weeks, suggesting that the AV conduction is able to partially recover from adult depletion of *Tbx3* [37]. AVN development is also specifically impaired after ventricular deletion of *Gata6* by direct inhibition of its target gene Id2 [60]. Inactivation of *Id2*, encoding a transcriptional repressor, causes abnormal development of the AVN by perturbing the cell cycle and Hcn4 expression. As mentioned above, the maturation of the AVN is also important in the development of AV conduction pathway and this step is disturbed in MyoR mutants by interfering with Cx30.2 expression through a direct interaction with Gata4 [61].

Defective development of the AVN is associated with AV electrical insulation disturbances or AV delay that cause AV accessory pathways or AV blocks.

Genetic Control of VCS Development

Before the emergence of a specific ventricular conduction system, cardiac conduction follows a unidirectional path from the inflow tract to the LV, RV and finally outflow tract (OFT). The mature activation pattern, characterized by two parallel apex-to-base activation waves, only emerges after ventricular septation and the development of a fast-conducting compartment in the subendocardial myocardium: the Cx40 positive trabeculae [11, 45, 46, 62, 63]. Subsequently, the gradual restriction of Cx40 expression to the future LBB, RBB and PF coincides with their maturation, eventually giving rise to the well-defined VCS [11, 14].

The proximal VCS – including AVB, RBB and LBB—arises from the primary interventricular ring (PIR) [64]. Interestingly, despite the close proximity and similarities of the BB and PF networks in the adult heart, the PF network does not originate from the PIR like the BB [64]. Instead the PF network arises from the $Cx40^+$ trabeculae [14, 65], which is consistent with the conductive function ensured by the trabeculae during development [45, 46, 62, 63]. Furthermore, the molecular signature of the trabeculae closely resembles that of mature PFs, and the progressive restriction of trabecular markers, including Cx40, Nppa, Irx3, Etv1, and Sema3a, during compaction reflects the maturation process of the PF network [11, 14, 65–69].

The timing of segregation also varies between the components of the VCS, occurring later in distal parts of the VCS. The AVB segregates the earliest, prior to E7.5, as shown by clonal analysis of SMA^+ cells [70]. The segregation to the LBB starts prior to E7.5 [70] and extends after E10.5 [64]. Finally, the PF network grows by recruitment of new conductive cells in successive waves throughout development [65, 71]. The first committed PFs can be observed as early as the linear heart tube stage (~E7.5) and likely define a scaffold for the formation of the mature VCS [65]. Interestingly, bipotent trabecular progenitors, which can give rise to both contractile CMs or be recruited to a PF fate, persist in the trabeculae until the time of birth. These bipotent progenitors are responsible for a second wave of PF recruitment that is key to increasing the complexity and density of the PF network [65]. After E14.5, the bipotent pool decreases dramatically and disappears by birth, marking the final segregation of contractile and conductive lineages [65]. Recently, scRNAseq at fetal stages (E16.5) identified two sub-clusters of immature PFs, one of which expresses intermediate levels of PF genes (Gja5, Etv1, Sema3a, etc.) [72]. This demonstrates heterogeneity in the level of differentiation among PFs, further confirming that conducting cells are recruited sequentially.

The early development of the LBB, like other derivatives from the PIR, is primarily regulated by Tbx3. At these early time points, Tbx3 refrains a fast conduction phenotype [57, 58], including through the inhibition of Cx40 expression. This is only around E14.5 that a gene regulatory network including Nkx2-5, Tbx5, Id2 is activated in the AVB and BB, favorizing the expression of the fast-conducting proteins including Cx40 and Nav1-5 [58, 73, 74]. Importantly, mutations of Tbx5 in these VCS components results in spontaneous ventricular tachycardia, lethal arrhythmias [73] and AV blocs [74].

The proximity of the VCS with the endocardium suggests an instructive role of this tissue in the development of the VCS. *Nrg1*, which is expressed by endocardial cells during development, is sufficient to convert immature CMs to a conductive phenotype without impacting proliferation [75, 76]. Nrg1 functions through the Ras MAPK pathway to activate Etv1, the most enriched TF in the developing and mature VCS [67, 76]. Consistent with the role of Nrg1 as a ventricular conduction system differentiation driver, its ectopic overactivation in embryonic CMs upregulates a subset of His-Purkinje–specific genes [77]. Additionally, other factors may predispose subendocardial CMs to be Nrg1 responsive [75], since, even under homogenous addition of Nrg1 in organ culture, conductive conversion occurs preferentially in the subendocardial myocardium. Furthermore, responsiveness to Nrg1 decreases as development proceeds accompanying the progressive segregation of conductive and contractile lineages. After conductive recruitment, Nrg1 appears to play a role in the late differentiation of PFs, as suggested by the conduction abnormalities observed following post-natal treatment with Nrg1-antagonist [76] (Fig. 1).

In parallel, Notch signaling also regulates conductive recruitment. Myocardial overactivation of Notch signaling, either in vivo throughout development, or transiently in neonatal CMs, is sufficient to induce the acquisition of a conductive phenotype by a subset of CMs without affecting their proliferation [53]. Notch induces the upregulation of both fast conducting proteins, Cx40 and Nav1-5, and nodal proteins, Cx30.2 and Hcn1, leading to the acquisition of PFs electrophysiological properties by former working CMs and

thereby, to VCS hyperplasia. Interestingly, only subendocardial CMs are responsive to Notch overactivation, suggesting that endocardial derived cues, such as Nrg1, could cooperate with Notch to pattern the VCS in subendocardial regions. Conversely, CHF1/Hey2, seems to repress conductive fate in the surrounding working myocardium and consistent with the observation of VCS hyperplasia in *CHF1/Hey2* KO mice [78]. Thus, Notch signaling regulates PFs patterning both by promoting a conductive fate within the VCS and by repressing conductive conversion in the neighboring working myocardium. Interestingly, conductive conversion under Notch overactivation, in the neonatal period, uncovers a conserved plasticity between conductive and contractile fates [53].

ETS Variant Transcription Factor 1 (Etv1) is directly activated by Nrg1 signaling and is necessary and sufficient to instruct a conductive phenotype. Indeed, *Etv1* KO mice display a hypoplastic VCS, especially in the mid and apical PF network where the number of characteristic ellipses is reduced [76]. Conversely, overexpression of *Etv1* in neonatal CMs or human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) is sufficient to induce a conductive phenotype [67, 76]. Etv1 also regulates the expression of *Cx40* and *Scn5a* in PFs, and thereby their electrophysiological properties. Thus, the Nrg1 – Etv1 axis functions as an upstream determinant of PF commitment and differentiation [67, 75, 76].

Part of the function of Etv1 appears to be mediated by the transcription factor Nkx2-5 [67, 76]. *Nkx2-5* expression is up-regulated in the developing His bundle, bundle branches and PFs in a spatiotemporal correspondence with the recruitment of conductive cells [56, 67, 72, 76, 79, 80]. This upregulation is necessary for a conversion to a conductive fate as attested by hypoplasia of the AVN, His bundle, LBB and PF network in *Nkx2-5* haploinsufficient mice [56, 59, 65, 81, 82]. Interestingly, the late recruitment of PFs is more sensitive to Nkx2-5 dosages than the early one. Thus, while the BB and the primitive scaffold are minorly affected in the *Nkx2-5* haploinsufficient mice, the mid and apical PF network is dramatically hypoplastic, with a reduction of two to three fold in the number of ellipses [56, 59, 65, 81, 82]. Surprisingly, the differentiation of the remaining PFs in *Nkx2-5* mutant doesn't seem to be affected, as their electrophysiological and Cx40 gap junctions number appears normal. However, other studies report that Nkx2-5 directly and indirectly – in part by upregulating of the homeodomain protein HOP – regulates a number of gap junctions and ion channels, including Cx40 [83]. Consistent with this, some cells with intermediate maturation have been identified in *Nkx2-5* haploinsufficient mice by their expression of EH-myomesin without Cx40 [81]. Finally, *Nkx2-5* is implicated in the maintenance of a conductive phenotype, as deletion of one copy of *Nkx2-5* at birth leads to a progressive loss of fast conduction proteins, including Cx40 and Hcn4 [84]. *Nkx2-5* expression in conductive cells is also regulated by the co-repression of the homeobox transcription factor prospero-related homeobox protein 1 (Prox1) and HDAC3 [85].

In cooperation with Nkx2-5, Tbx5 and Irx3 orchestrate the recruitment and specification of PFs and can thus partially compensate for low levels of *Nkx2-5* in some conditions (Fig. 1).

Irx3, which is expressed in the immature proximal VCS and in a subset of trabeculae during development, is restricted to the VCS of the adult heart. Similar to *Nkx2-5* haploinsufficient mice, *Irx3* KO mice develop hypoplastic bundle branches and PF network, defects that manifest mainly in the postnatal period [86]. Furthermore, *Irx3* regulates gap junction expression in PFs by indirectly upregulating *Cx40* and directly inhibiting *Cx43* expression in competition with *Nkx2-5* [68]. As a result, *Irx3* KO mice express only half of the WT levels of Cx40 in their remaining VCS, while their LBB ectopically express *Cx43*, resulting in abnormal contact between the LBB and surrounding myocardium.

Similar to *Nkx2-5, Tbx5* has a pleiotropic role in cardiac development causing embryonic lethality in $Tbx5^{-/-}$ mice and multiple cardiac defects in Tbx5 haploinsufficient mice [87]. *Tbx5* expression is enriched in the mature VCS compared to the surrounding myocardium and maximal levels of *Tbx5* are required for the recruitment of conductive cells in the His bundle and bundle branches [67, 74, 76]. Consequently, *Tbx5* haploinsufficient mice have a hypoplastic His bundle, and immature bundle branches. In addition, *Tbx5* cooperates with *Nkx2-5* to synergistically activate *Cx40* and *Nppa* expression [87]. To date, the role of *Tbx5* specifically in PFs has not been investigated. The cooperative role of *Nkx2-5, Tbx5* and *Irx3* is illustrated by their direct physical interactions and the presence of neighboring binding sites for all three TFs in the regulatory regions of a large number of target genes including cyclin-dependent kinase inhibitors [86]. Furthermore, compensatory upregulation of *Nkx2-5* and *Tbx5* has been reported in $Irx3^{-/-}$ mice, while double or triple KO mice show increasing defects in VCS patterning [86, 88].

miR-1, a direct target of *Nkx2-5*, is the most abundant microRNA in the postnatal heart and plays a pleiotropic role in regulating CM electrophysiology, proliferation and VCS development [89–91]. During development, miR-1 represses CM proliferation through a Cdk6 /Pocket protein axis, resulting in control of myocardial and VCS growth [90]. Consequently, premature expression of miR-1 in CMs results in reduced CM proliferation and hypoplasia of the PF network. Conversely, Pocket proteins 3KO ($p107^{-/-}$, $p130^{-/-}$, $Rb^{del/del}$) mice have hypertrabeculated ventricles due to uncontrolled CM proliferation in both the compact layer and trabeculae resulting in thickening of the His Purkinje system [92]. These results show that increasing or decreasing the

proliferation of the trabeculae, which contain the pool of PF progenitors, is sufficient to increase or decrease the production of PFs [90, 92].

Hand1 is expressed in the LV from E8.5, and, together with Hand2, regulates trabecular identity and represses trabeculae proliferation [93]. Mice lacking *Hand1* expression in the LV ($Hand1^{\Delta LV/\Delta LV}$) develop a hyperplastic VCS, which may be due to increased trabecular proliferation [94]. However, both the left and right VCS are hyperplastic in $Hand1^{\Delta LV/\Delta LV}$ mice, even if Hand1 is only expressed in the LV of developing hearts. This, together with the observation that *Hand1* is expressed in the His bundle, RBB, LBB, left PFs and, potentially right PFs in mature hearts [94], suggests that Hand1 may have an additional role in PF recruitment or differentiation (Fig. 1).

PFs specifically express three proteins of the immunoglobulin family: *Cntn2, Ncam-1 and Alcam* [95, 96]. LOF models indicate that *Ncam-1* KO mice have a hypoplastic VCS, especially at the apex, similar to of *Etv1* KO, *Irx3* KO or *Nkx2-5* heterozygous mice, supporting a role for Ncam-1 in VCS patterning. In addition, polysialylated Ncam-1 is essential for PF differentiation by controlling the localization of Cntn2, Ncam-1, Cx40, components of the desmosomes and adherent junctions at the intercalated discs. It is interesting that an adhesion molecule can play a role in the patterning of the VCS and may reveal a feedback loop between electromechanical coupling in the PFs and PF commitment and differentiation. Furthermore, this may explain why some mutant mice develop hypoplasia of the VCS specifically in the neonatal period [81, 86], when the maturation of the intercalated discs takes place (Fig. 1).

Novel Insights Into the Differentiation Trajectories of CCS

Recent advancements in single-cell transcriptomic technologies, including single-nucleus RNA sequencing (snRNA-seq) and spatial transcriptomics (STx), have significantly enhanced our understanding of the transcriptomic landscape of the CCS, improving our ability to capture cell-state heterogeneity and define developmental trajectories underlying CCS differentiation. In 2019, single-cell RNA-seq (scRNA-seq) analysis on microdissected mouse embryonic hearts, including the sinoatrial node (SAN), atrioventricular node (AVN), His bundle, and Purkinje fibers (PF), provided the first comprehensive single-cell transcriptional profiling of the developing CCS [72]. Unsupervised cell clustering along with gene enrichment analysis identified novel conductive cell markers (*Igfbp5, Cpne5, Smoc2, Rgs6, Ntm*) and revealed the existence of rare conduction cell subtypes in each CCS component [72]. Direct comparison of embryonic and postnatal scRNA-seq datasets with postnatal stages highlighted high transcriptional similarities between fetal and neonatal stages suggesting that CCS cell fate, particularly for the SAN, cAVN and proximal VCS, may largely occur by E16.5 [97]. Cell-state heterogeneity of the CCS, particularly of the SAN, has also been reported in the adult murine heart, including a core cell cluster functionally related to the regulation of heart rate. Among the canonical SAN markers, *Vsnl1* has been identified as a novel SAN gene whose expression regulates the beating rate of hiPSC-CMs and mouse hearts [98].

The combination of single-cell analysis with unbiased, transcriptome-wide spatial gene expression information is now opening new horizons in understanding cardiac cell states in relation to their anatomic location within the different cardiac compartments. Integration of scRNA-seq and spatially resolved transcriptomic analysis in the developing murine heart has identified a novel cardiomyocyte population expressing *dopamine beta-hydroxylase* (Dbh + CMs), which is transcriptionally and functionally associated with the CCS in developing and mature murine hearts [99].

More recently, spatiotemporal resolved scRNA-seq analysis of the developing human heart has provided novel insights into the differentiation of the CCS [100, 101]. In particular, a detailed molecular characterization of conductive cardiomyocytes in the developing CCS compartments highlights their distinct electrophysiological properties in close relationship with spatial and functional associations with other cell types, including specialized fibroblast cell states in the nodes, endocardial cells in the ventricular conduction system, and neurons in the developing autonomic innervation [100]. The increasing generation of new multiomic datasets in both, CCS development and disease, will be instrumental for gaining novel insights into CCS development and translate this information to identify novel therapeutic approaches to treat cardiac arrhythmias.

Conclusions

The CCS is a complex tissue with its different compartments having specific functions and thus, specific electrophysiological properties in the adult. Each compartment arises from different populations and its development is regulated by distinct gene regulatory networks. The development of the CCS involves the proliferation of progenitors, the recruitment to a conductive fate, the acquisition and the maintenance of conductive properties. Though these processes are in theory independent, many factors have a pleiotropic role in the regulation of several of these steps. Dysregulation of any of these processes can result in mispatterning of one or several components of the CCS, such as hypoplasia. Importantly, all model of CCS hypoplasia—$Nkx2\text{-}5^{+/-}$, $Tbx5^{+/-}$, $Irx3^{-/-}$, $Id2^{-/-}$, $Etv1^{-/-}$, $Ncam\text{-}1^{-/-}$ mice—present slowed conduction in the affected compartment(s) [59, 68, 74, 76,

81, 86, 96, 102–104]. Moreover, abnormal electrophysiological properties of conductive cells, caused by abnormal gap junction or ion channel content, can also, in combination with patterning defects or alone, result in perturbed conduction [84, 104, 105].

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Competing interests The authors declare no competing interests.

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