Review

The development of specialised cardiac muscle cells within a vertebrate heart requires a specialised regulatory network

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Posted Date: 5 July 2023

doi: 10.20944/preprints202307.0335.v1

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Review

The Development of Specialised Cardiac Muscle Cells within a Vertebrate Heart Requires a Specialised Regulatory Network

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Abstract: Heart is composed of muscle cells called cardiomyocytes, including a specialized population, named pacemaker cells, that form the Cardiac Conduction System (CCS), responsible for generating the action potential dictating heart contractions. Failure of the CCS system leads to cardiac arrhythmias requiring complicated therapies and often surgical implantation of electrical pacemakers. However, recent research focusses on development of novel therapies using biological pacemakers aiming to substitute electrical devices. While most signalling pathways and transcription factors involved in the development of the pacemaker cells are known, the upstream regulatory networks need to be predicted through computer-based databases, mathematical modelling as well as functional testing of the regulatory elements *in vivo*, indicating the need for further research. Here we summarise the current knowledge about the vertebrate myocardial CCS system and development of the pacemaker cells and emphasise areas of future research to clarify the regulation of muscle pacemaker cells and ease development of biological therapies.

Keywords: cardiac conduction system; pacemaker; gene regulatory network

1. Contracting Cardiac Muscle

The heart is a muscular organ that pumps to circulate the blood throughout the body with the ability to initiate and coordinate electrical impulses to begin contractions of the atria and ventricles. The production of electrical impulses by the heart is regulated by the Cardiac Conduction System (CCS), specifically the Sinoatrial Node (SAN) (Mohan et al., 2017; van Weerd and Christoffels, 2016). These contractions are accomplished by the well-timed coordination of the fast and slow components of the CCS, which are of myogenic origin (Cheng et al., 1999; Meilhac et al., 2004). In higher vertebrates, the CCS is classified into the SAN or the primary pacemaker site, atrioventricular node (AVN), the secondary pacemaker site, and the "wiring" of the ventricles, the His-Purkinje fibres (Davies, 1942; Mohan et al., 2017). Electrical impulses are first generated in the SAN, located between the superior vena cava and the right atrium, and are rapidly propagated through the cardiomyocytes (CMs) of the atrium, resulting in an atrial contraction (Kennedy et al., 2016). After the initiation of an atrial contraction, the electrical impulses reach the slow conducting tissues of the secondary pacemaker site, the AVN, located within the atrioventricular septum, causing a delay before leading on to the His-Purkinje fibres, resulting in a ventricular contraction (Moorman et al., 1998; van Weerd and Christoffels, 2016) (Figure 1).

Within a human embryo, the first signs of heart muscle contraction typically begin around embryonic day 22 during the third week of gestation, when the first heart field develops into the heart tube (Tan and Lewandowski, 2020). Comparatively, contraction of the heart muscle in mice begins earlier around embryonic days E8 to E9 (Andres-Delgado and Mercader, 2016; Lindsey et al., 2014). However, in zebrafish, with a different anatomical structure of the heart muscle compared to human, mouse or chicken, contraction begins at 22 hours post fertilisation with contraction beginning in chicken at around HH10 to HH11 (Martinsen, 2005; Wittig and Munsterberg, 2020; Xia et al., 2020). Once the heart tube fully develops, the rate of contraction of the heart muscle increases with the heart

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forming the ability to pump blood efficiently throughout the body (Kennedy et al., 2016; Moorman et al., 1998; van Weerd and Christoffels, 2016).

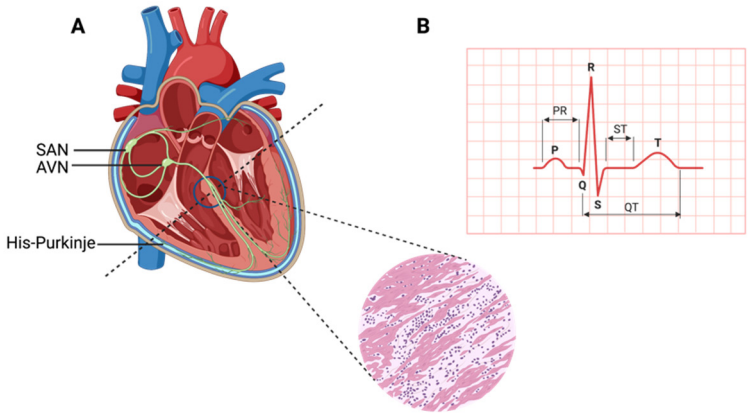

Figure 1. Cardiac contraction and histology of cardiac muscle. (**A**) Schematic representation of the components of the cardiac conduction system (CCS) in a human heart. In the inset picture, a cross-section of the human heart muscle is shown, with binucleated cardiomyocyte. The various components of the CCS (in green) are labelled: Sinoatrial node (SAN) located at the junction of the superior caval vein and right atrium, generates the impulse that then travels to the atrioventricular node (AVN). Propagation occurs through the left and right bundle branches of His-Purkinje leading to ventricular contraction. (**B**) An electrocardiogram representing the recording of the electrical activity of the heart. The upper chambers of the heart (atria) begin to beat when the first wave of the ECG, labelled P, appears. The lower chambers of the heart (ventricles) are represented by the QRS complex as electrical current flow. The electrical current spreads back over the ventricles in the opposite direction during the recovery phase, which is represented by the T wave.

Studies from mammalian, avian and fish model systems have shown that each CCS component consists of a specialized group of CMs with distinctive morphological and electrophysiological properties and transcriptional profiles (Anderson et al., 2009). The dysfunction of any of CCS components can lead to cardiac arrhythmias like Brugada syndrome, long QT syndrome and sudden cardiac death (Barbuti et al., 2007; Cohle et al., 2002). Some of these arrhythmias complicate therapy for congenital cardiac conditions. As a result, comprehension of the CCS gene regulatory work is essential for the creation of therapeutics for cardiac arrhythmias.

Treatment of various types of arrhythmias need the installation of electrical pacemakers. Current and older generations of electrical pacemakers have been known to have a variety of limitations to their design and function including issues with battery life, generator failure and risk of infection with materials used (Alasti et al., 2018; Cingolani et al., 2017). The limitations and complications with electrical pacemakers led to biological pacemakers being researched and developed to be used in alternative (Cingolani et al., 2017). Although heart development has been studied for more than a century, little is known about the development of its pacemaker cells mainly due to the absence of a complete understanding of the genetic mechanism regulating its development. The development of biological pacemakers needs the translation of already known knowledge of the signaling pathways, transcription factors, and their gene regulatory networks to increase the reprogramming efforts.

2. Key Transcription factors involved in Cardiac Conduction System development

Cardiac Conduction System development and homeostasis is reliant on transcriptional and regulatory networks that are embryonic-stage-dependent, dose-dependent, and tissue-dependent (Li et al., 2022; Liang et al., 2020; Schram et al., 2002). A cascade of transcription factors including SHOX2, BMP4, SCN5A, TBX3, TBX5, NKX2-5, ID2 and more has been established as instrumental to this spatial divergence in myocytes development, as will be evidenced in the sub-sections below.

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2.1. Short stature homeobox 2 (SHOX2) and bone morphogenic protein 4 (BMP4)

SHOX2 and BMP4 are important for SAN formation (Liang et al., 2015; van Weerd and Christoffels, 2016); van Weerd and Christoffels, 2016). Dysfunction of the SAN can lead to a variety of cardiac arrhythmias including bradycardic arrhythmias (Hu et al., 2018). SHOX2 transcription factor is essential for SAN formation and differentiation with SHOX2 being widely expressed throughout the body including the heart muscle (Hu et al., 2018). BMPs are a group of signalling molecules that belongs to the Transforming Growth Factor β superfamily of proteins that have a key role in pacemaker development. The key role of BMP4 in embryonic heart development is the promotion of fibroblast reprogramming to cardiomyocytes with pacemaker activity (Efe et al., 2011). BMP4 is also a target of SHOX2 with the expression of both SHOX2 and BMP4 overlapping (Puskaric et al., 2010). One of the main functions of BMP4 is its role in the differentiation of the cardiac pacemaker cells (Wu et al., 2019). A series of epistatic genetic experiments in Xenopus have elucidated that SHOX2 interacts directly with BMP4 promoter and the expression patterns of BMP4 and SHOX2 overlap in the SAN during embryonic development (Puskaric et al., 2010).

2.2. T-box transcription factor 5 (TBX5), NK2 Homeobox 5 (NDX2-5) and Inhibitor of DNA binding 2 (ID2)

TBX5, NKX2-5 and ID2 are essential for atrioventricular bundle and bundle branch development (Moskowitz et al., 2007; van Weerd and Christoffels, 2016). TBX5 has a variety of roles throughout the body but specifically has main function within cardiac development (Moskowitz et al., 2007). Mutations of the TBX5 transcription factor is known to lead to cardiac defects at the cardiac septa and cardiac conduction system. Within early embryonic cardiac development, TBX5 works as a transcriptional activator for genes working in the maturation of cardiomyocytes (Moskowitz et al., 2007). Later in cardiac development, TBX5 role changes and focusses on the structure of the cardiac conduction system as well as the maintenance of cardiomyocyte maturation (Moskowitz et al., 2007). NKX2-5 is a cardiac homeobox transcription factor that is expressed throughout the cardiac system (Nakashima et al., 2014). Mutations within NKX2-5 lead to cardiac defects and atrioventricular conduction abnormalities. Throughout cardiac development, NKX2-5 has been found to have roles in the regulation of the working and conducting myocytes within the atria whilst working alongside the Notch signalling pathway (Nakashima et al., 2014). ID2 is a cardiac transcription factor with expression being detected around the neural crest, inflow and outflow tract as well as within the neurons around the aorta and pulmonary artery (Fraidenraich et al., 2004; Hu et al., 2019). Later in development ID2 is expressed in the atrioventricular bundle at E12.5 and within the bundle branches around E16.5 (Hu et al., 2019; Moskowitz et al., 2007).

2.3. T-Box Transcription Factor 3 (TBX3)

TBX3 is expressed in the cardiac conduction system of the heart muscle and is essential for repressing atrial differentiation (Wiese et al., 2008). Various studies have identified noncoding variants near expression of TBX3 that is linked to PR interval and QRS duration, suggesting that changes to TBX3 expression has the ability to affect the function of the atrioventricular conduction system (Eif et al., 2018; Harst et al., 2016; Mohan et al., 2017; Pfeufer et al., 2010; Setten et al., 2018; Sotoodehnia et al., 2010; Verweij et al., 2014).

2.4. T-box transcription factor 18 (TBX18)

TBX18 has key roles in heart muscle development, particularly in the formation and structure of the SAN (Greulich et al., 2011; Wu et al., 2019). Furthermore, expression of TBX18 is essential for early SAN specification and specifically produces pacemaker activity within early development of embryonic heart muscle formation (Cho, 2015; McNally and svensson, 2009; Wiese et al., 2008). Additionally, one study successfully converted ventricular myocytes to induced pacemaker cells within postnatal rats via TBX18 expression potentially providing a novel method for biological pacemakers (Kapoor et al., 2013).

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2.5. ISLET-1 (ISL1)

ISL1 is a transcription factor with roles in multiple organs during embryonic development with roles within cardiac development specifically as a marker for second heart field progenitors (Ren et al., 2021). ISL1 expression is detected as early as E7 in mouse heart development however, expression of ISL1 changes throughout development with expression being seen in the SAN from postnatal to adulthood (Zhou et al., 2019).

2.6. GATA4

GATA4 is a regulator of cardiomyocyte proliferation, and differentiation with expression being high until birth but remains detectable in all cardiomyocytes (Whitcomb et al., 2020). Mutations with GATA4 results in cardia bifida which signifies the function of GATA4 in early heart formation (Molkentin et al., 1997; Whitcomb et al., 2020). GATA6 is a regulator of SAN development with mutations with GATA6 leading to dysfunction with SAN patterning and size leading to arrhythmias (Gharibeh et al., 2021).

2.7. HAND1

HAND1 plays an essential role in the specification and differentiation of embryonic structures including the cardiac muscle of the heart (Zheng et al., 2021). HAND1 is an essential regulator for cardiac precursor cell fate decision and morphogenesis regulated by the signalling pathway, BMP (Firulli et al., 2020). Mutations within HAND1 have been shown to be associated with cogenital heart disease (Firulli et al., 2014; Vincentz et al., 2017). Further research found that BMP can activate BMP signalling-HAND1 regulation within heart muscle development (Zheng et al., 2021).

2.8. IRX3

IRX3 is essential for the regulation of rapid electrical propagation in the ventricular conduction system by transcription of Cx40 and Cx43 (Kim et al., 2016). Development of the ventricular conduction system is regulated by the activation of transcription factors including NKX2-5, TBX3, TBX5, and ID2 (Bakker et al., 2008; Briggs et al., 2008; Hoogaars et al., 2004; Jay et al., 2004; Kim et al., 2016; Moskowitz et al., 2007; Moskowitz et al., 2004). Loss of the transcription factors leads to many cardiac defects specifically the loss of NKX2-5 and TBX5 leading to the increased chance of developing arrhythmias (Kim et al., 2016). IRX5 is expressed in a gradient in the ventricular myocardium with the epicardium having the lowest expression and the endocardium having the highest expression (Munshi, 2012). IRX5 mutations leads to increased chance of developing arrhythmias due to having abnormal repolarisation within the ventricular conduction system in the absence of a homeostatic Kv4.2 gradient (Costantini et al., 2005; Munshi, 2012).

3. Key Signalling pathways involved in CCS development

The conserved signalling pathways that have been found to be crucial for CCS specialization are Notch, BMP, Wnt, and NKX2-5 (Hoogaars et al., 2004; Liang et al., 2015; Luxan et al., 2016; Wang et al., 2020).

3.1. Notch signalling

Notch signalling pathway is a conserved pathway that has critical roles in the regulation of cell fate specification and their differentiation, and tissue patterning (Luxan et al., 2016; Nakano et al., 2018; Rentschler et al., 2012). Similarly, a knock-out study revealed Notch1 to be lethal around E9.5 to E11.5 as it controls the development of the sinus venous valve and the SAN by coordinating myocardial Wnt and NRG1 signalling functions (Luxan et al., 2016; Wang et al., 2020).

3.2. BMP signalling pathway

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The BMP (bone morphogenetic protein) signalling pathway is involved in the differentiation of the sinoatrial node (SAN) and the atrioventricular node (AVN) and regulates cardiac progenitor development (Wang et al., 2010). Regulation of the BMP signalling pathway is coordinated by the SMAD proteins with the BMPs being associated with the TGFβ superfamily (Callis et al., 2005). The TGFβ pathway is involved in processes throughout the heart muscle as well as in the formation and patterning of the cardiac conduction system (Hoogaars et al., 2004; Yousefi et al., 2020). Various studies have shown that TGFβ signalling is essential for heart muscle development specifically TGFβ1, 2, and 3 being expressed in specific regions and stages of the cardiac conduction system development of the heart muscle (Ramos-Mondragon et al., 2008).

3.3. Wnt signalling

The Wnt signalling pathway regulates the proliferation and differentiation of cardiac progenitor cells during cardiac development and the formation of the conduction system. Recently, Liang and colleagues have shown that canonical Wnt signalling promotes pacemaker cell specification of cardiac mesodermal cells derived from mouse and human embryonic stem cells (Liang et al., 2020). They have shown that one of the key canonical Wnt/β-catenin ligand, Wnt3a, enhances the expression of a chamber of cardiomyocyte genes NKX2-5. This rises the number of pacemaker-like myocytes while reducing cardiac troponin T-positive pan-cardiac differentiation (Liang et al., 2020).

The signalling pathways involved with the development of the cardiac conduction system of the heart muscle interact with each other and with various other factors to regulate the development and function of the CCS. Reprogramming efforts in human induced pluripotent stem cells have shown the impact of other signalling pathways like FGF and retinoic acid which reprogram the cardiac mesoderm to generate SAN-like cells (Liu et al., 2020b). Transcriptome analysis of mouse and human sinoatrial node cells and sinoatrial ring (SAR) in zebrafish reveals a conserved genetic program (Burkhard and Bakkers, 2018; Efe et al., 2011; Liang et al., 2020; Minhas et al., 2021; Puskaric et al., 2010; van Eif et al., 2019) (Liu et al., 2020a).

4. The genetic network of CCS development

A unique gene expression mechanism enables cardiac pacemaker cells in the SAN to fire autonomously and initiate the heartbeat. The CCS is evolutionarily conserved in the building plan of the heart, and this indicates that the cellular and molecular mechanisms that drive the formation of pacemaker tissues are almost similar among vertebrates. Studies have shown that mammalian pacemaker CMs exhibit typical pacemaker action potentials and express molecular markers such as *Isl1, Shox2* and *Hcn4* (Blaschke et al., 2007; Minhas et al., 2021; Tessadori et al., 2012; Vedantham et al., 2015). These mammalian genes are conserved in zebrafish and other teleost species. Knocking down of these genes in zebrafish leads to bradycardia, a phenotype indicating defects in cardiac pacemaker activity, further supporting their conserved roles for regulating pacemaker development in zebrafish (Blaschke et al., 2007; de Pater et al., 2009; Tessadori et al., 2012). The transcription factors (TFs) and the signaling pathways that control CCS specification constitute a gene regulatory network, and at the core of these networks are the *cis*-acting regulatory regions that are bound by TFs. Recent studies in mice have reported an Isl1 specific enhancer which has not been identified in zebrafish (Galang et al., 2020). However, there is limited understanding of the underlying gene regulatory network of these critical cells that are responsible for the electrical conduction of the heart.

For better understanding of such GRNs, the identification of *cis*-regulatory modules involved in the development of the cardiac conduction system will pave the way to elucidate molecular mechanisms underlying their regulation of expression. Tissue-specific gene expression obliges long-range regulatory regions, such as enhancers, which dictate the precisely spatial-temporal and dosage-dependent expression of their target genes (Minhas et al., 2019). The availability of publicly available genomics data, well-established protocols for chromosomal conformation capture followed by next-generation sequencing in isolated hearts (3C, 4C-Seq, 5C, Hi-Seq), or derived methods like FAIRE-Seq and ATAC-Seq, single-cell sequencing, multiple genome-wide Chip-seq datasets and evolutionary conservation studies across various vertebrate model species (for example mice, chicken

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or zebrafish) can identify multiple regulatory landscapes which act as cardiac conduction-specific enhancers (Moskowitz et al., 2007). These identified enhancers can then be functionally tested *in vivo,* and mutants generated using CRISPR/Cas9 (Figure 2). Mutant generation by genome-editing techniques like CRISPR/Cas9 can be used to: (1) study the role of the identified enhancers in development of CCS, and (2) gene expression patterns of the regulated genes can provide insights into diseases like cardiac arrythmias.

Thus, the GRN for key TFs involved in CCS like *NKX2-5*, TBX3, *TBX5*, *ISL1*, *GATA4*, *GATA6*, *HAND1*, *SHOX2*, IRX3 and IRX5 needs to be investigated systematically to build the CCS-specific gene regulatory network.

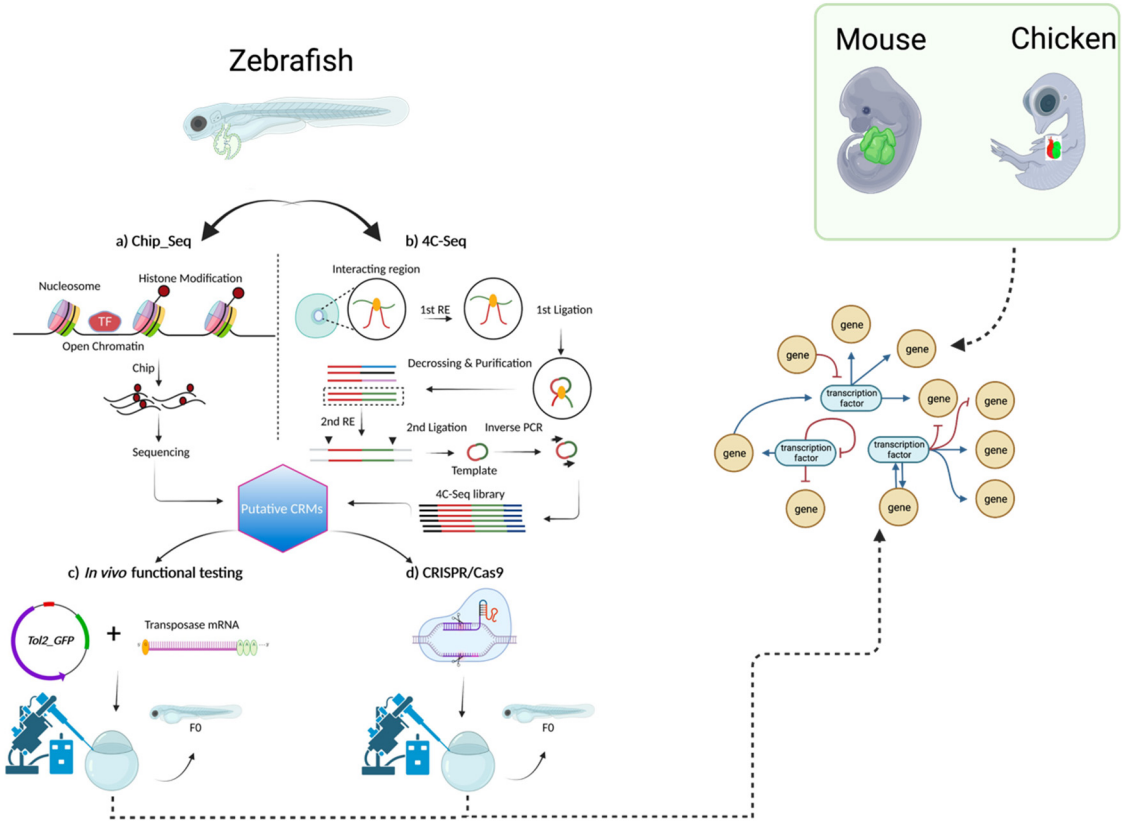

Figure 2. Proposed methodology to further investigate CCS-specific enhancers leading to building an informative GRN. Schematic of methods that can be used to identify cis-regulatory modules (CRMs) using isolated hearts of various key developmental models (zebrafish, mice or chicken). For each model, in this case zebrafish is shown, various chromatin capture methods like ChIP-Seq or 4C-Seq can be employed to get a list of putative CRMs. These CRMs can be functionally tested and investigated further using genome-editing techniques like CRISPR/Cas9. A functionally validated GRN can then be generated by intersecting data obtained from several developmental models.

5. Conclusion and future directions

Building a CCS-Specific GRNs needs computational predictions through publicly accessible databases, mathematically modelling, followed by functional testing of the regulatory elements *in vivo.* This will be further validated by knock-out studies. This needs a lot of seriously multi-disciplinary collaborative work (including work from system biologists, developmental biologists, and molecular biologists, and computational experts) and funding to work in parallel to work and highlight and predict the important genes and their putative regulatory regions to test them functionally.

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