Modeling of the cardiac sympathetic nervous system and the contribution of epicardium-derived cells

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General Introduction and thesis outline

The Development of a Functional Heart

A normal developed heart is a complex self-excitation organ that is composed of four chambers (two atria and two ventricles) [1]. The wall of the four-chamber heart consists of three layers: the endocardium, the myocardium and the epicardium [2]. The cardiac conduction system[1], consisting of myocardium cells with a distinct molecular signature from the working myocardium, generates and coordinates the electric signal by which mechanical activation by the sequential contraction of first the atria followed by the ventricles, is established [3, 4]. A functional heart is able to adapt to environmental changes (e.g. level of oxygen or stress) and body conditions (e.g. resting condition versus exercise) via adjusting the contraction rate and force. These cardiac adaptations are achieved by regulation of adrenaline release from the adrenal gland as well as by a controlled coordination of the parasympathetic and sympathetic cardiac activation, referred to as cardiac autonomic nervous system regulation [5, 6].

Development of the Heart and the Essential Role of Epicardium. During development, cardiogenesis begins with the formation of the cardiac crescent by E7.5 in mice and day 15 in human embryos [7, 8]. At this early stage two cardiac heart fields fuse in the ventral midline and give rise to the cardiac crescent, which will remodel into a single tube structure [8]. This primitive cardiac tube initially consists of two layers: the inner endocardium, which is a sheet of specialized epithelial cells that is in contact with the first blood cells, and the outer myocardium, which is a layer of cardiac muscle cells that starts to beat at day 22 in human embryos. Soon after the heart tube formation, around E8 in mice and day 23 in human embryos, the tube starts to elongate loop with an active blood circulation at E10 in mice and day 28 in human [7-10]. Subsequently, after cardiac looping and septation, a four-chamber heart with recognizable atrial and ventricular compartments is formed [11, 12].

The epicardium, the third layer, is formed after the cardiac looping. The epicardium originates from the so-called proepicardial organ (PEO) (**Figure 1**), which is an cauliflower-like cluster of extra-cardiac cells situated at the venous pole of the heart. The PEO is an outgrowth of the coelomic mesothelium at the ventro-caudal base of the developing heart around E8.5 in the mouse, and is composed of heterogeneous cell populations [13]. A subgroup of PEO cells, expressing Wilms' tumor 1 (WT1) transcription factor, T-box transcription factor 18 (Tbx18) and the basic helix-loop-helix Transcription Factor 21 (Tcf21), migrates along the inflow tract,

[1] Sinoatrial Node (SAN), Atrioventricular Node (AVN), Atrioventricular Bundle (AVB), left and right Bundle Branches (BBs), Peripheral Ventricular Conduction System (PVCS).

proliferates and gradually spreads to cover the heart to form an epithelial outer layer (**Figure 1**).

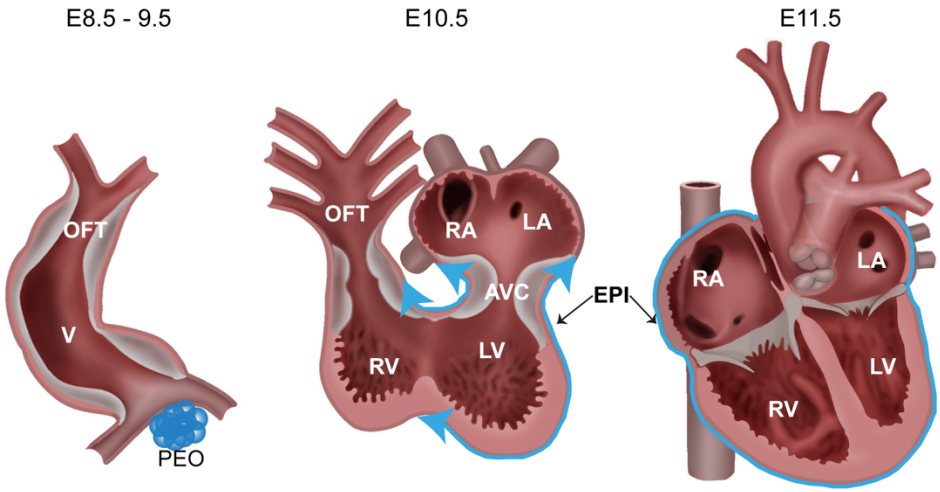

Figure 1. The proepicardial organ (PEO) and the development of epicardium. The expression of WT1 is indicated in blue. OFT, outflow tract; V, ventricle; PEO, proepicardium; RA, right atrium; RV, right ventricle; AVC, atrioventricular cushion; EPI, epicardium; LA, left atrium; LV, left ventricle.

Although initially consisting of only one cell layer, the epicardium plays an essential and critical role in cardiac development. Once the epicardium envelops the entire heart, part of the epicardial cells lose cell-cell contact, initiating the process of epithelial-to-mesenchymal transition (EMT), where after they are referred to as epicardium-derived cells (EPDCs) [14, 15]. EPDCs are deposited into the subepicardial space and subsequently migrate into the myocardium where they differentiate into multiple cardiac cells types, including interstitial fibroblasts, adventitial fibroblasts and smooth muscle cells of the coronary vessels [16-18] (**Figure 2**). Whether EPDC also differentiation into coronary endothelial cells and cardiomyocytes has been largely debated. Moreover, the role of the epicardium is not limited to a structural contribution to the cardiac lineages. EPDCs also possess paracrine functions critical for myocardial growth, as well as for coronary vessel [19-21]. As the heart matures, the epicardium becomes quiescent [17]. Inhibition of epicardium formation during cardiogenesis by mechanically removing the PEO, by obstructing the outgrowth of the PEO in the chicken embryo or by genetic knockdown methods in mice embryos results in (amongst others) a thin, noncompact ventricular myocardium and incomplete formation of coronary vasculature [22-24], which emphasizes the essential role of epicardium and EPDCs in heart development.

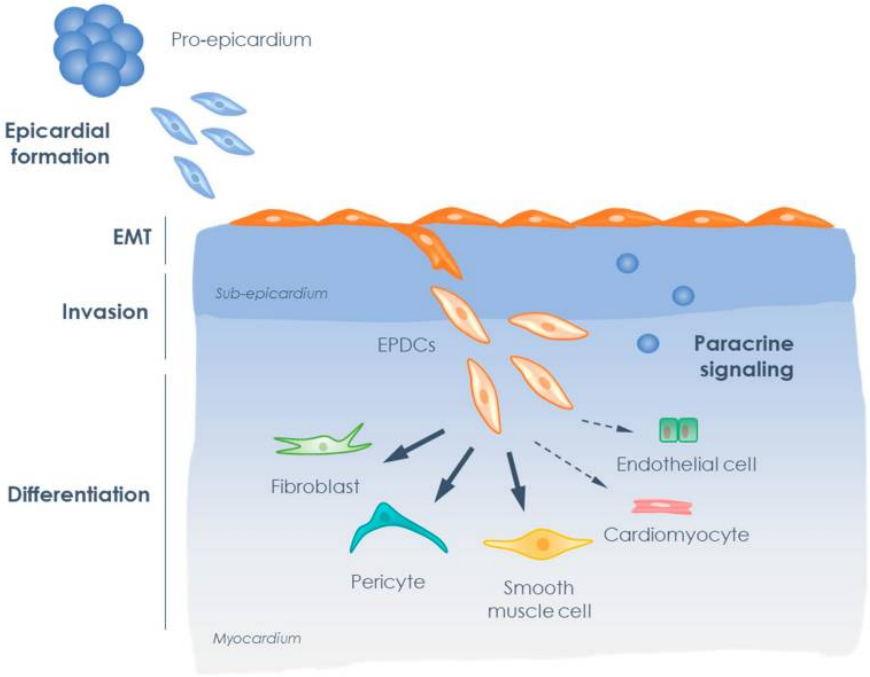

Figure 2. Schematic overview of epicardial development and differentiation during development. The figure is from Dronkers et al. Biomolecules. 2020

Cardiac Autonomic Nervous System and Its Development. A fully developed contractile heart is controlled by the autonomic nervous system (parasympathetic and sympathetic) to adapt to the body's demand for oxygenated blood under different conditions. The autonomic nervous system regulates the heart rate, blood pressure, respiration rate, body temperature, transpiration, as well as visceral activation to maintain homeostasis [25-27]. The cardiac autonomic nervous system is centrally coordinated by the thalamus at the top of the brain stem and the medulla oblongata within the brain stem. The peripheral autonomic system can be divided into a sympathetic and parasympathetic part. The sympathetic and parasympathetic system contain efferent (motor neurons) elements of the visceral peripheral nervous system (**Figure 3**).

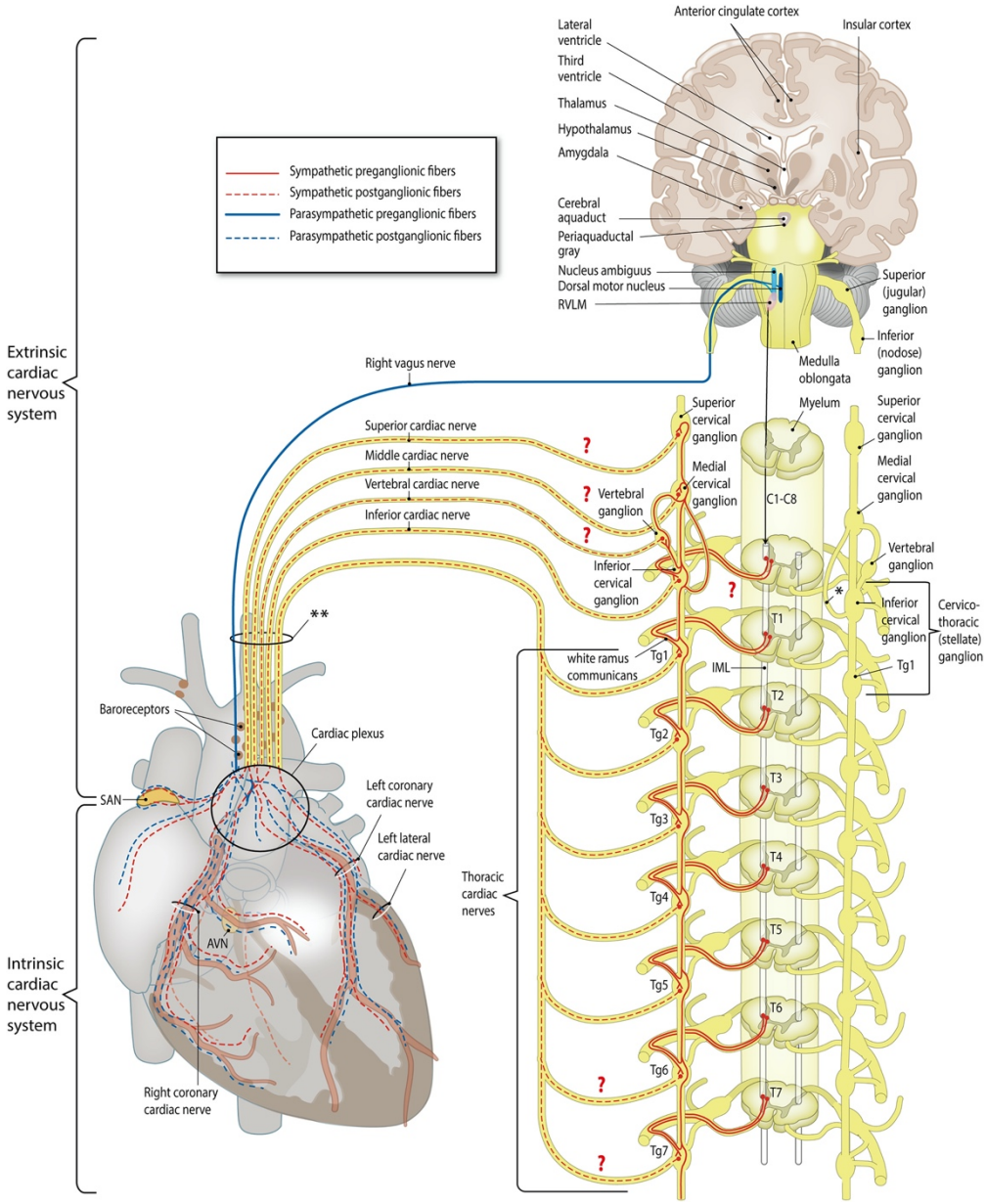

Figure 3. Schematic drawing of the cardiac autonomic nervous system. The figure is from Wink et al. Auton Neurosci. 2020.

The efferent portion of cardiac sympathetic nervous system includes short preganglionic nerves, cardiac sympathetic ganglia (cervical ganglia, stellate ganglia as well as upper thoracic ganglia of the sympathetic chain) and long postganglionic sympathetic nerves [25, 28] **(Figure 3)**. Acetylcholine is used as pre-ganglionic sympathetic neurotransmitter, while norepinephrine (NE) is the post-ganglionic sympathetic neurotransmitter of the sympathetic nervous system. Activation of post-ganglionic nerves results in the release of NE from sympathetic nerve terminals to the synapse with cardiomyocytes. NE binds to the post-synaptic adrenergic beta-1 receptor in the cardiomyocytes of the working myocardium and conduction system, resulting in an increased heart rate, increased contractility as well as an increased AVN conduction velocity [5]. Tyrosine hydroxylase (TH) catalyzes the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), which can, through a series of downstream enzymatic reactions, be processed into dopamine and be further altered into NE [29-32]. Therefore, in the peripheral nervous system, TH can be used to identify sympathetic neurons.

In contrast, the efferent part of the cardiac parasympathetic nervous system is composed of long preganglionic nerves from the central nervous system, cardiac ganglia that are situated on the surfaced of the heart (centers where the majority of cardiac parasympathetic neurons interconnect), and short postganglionic parasympathetic nerve fibers [25, 28] **(Figure 3)**. Acetylcholine is the neurotransmitter for both pre- and postganglionic parasympathetic nerves. Acetylcholine inhibits the contraction of cardiomyocytes by activating muscarinic receptors (M2) in cardiomyocytes [5]. The synthesis of acetylcholine in parasympathetic neurons is achieved by choline acetyltransferase (ChAT) that catalyzes the synthesis of acetylcholine from choline and acetyl-COA [5]. Therefore, ChAT is usually used as a marker for postganglionic parasympathetic neurons in the peripheral nervous system.

The development of peripheral cardiac autonomic nervous system is a relatively late phenomenon. Sympathetic ganglia are formed as a result of trunk neural crest cells that migrate, proliferate and differentiate into mature sympathetic neurons [33, 34]. Parasympathetic neurons in cardiac ganglia, however, are derived from cardiac neural crest cells, which also participate in the septation of the outflow tract of the heart [34-37].

Cardiac innervation patterning is a very complex process, which is directed and modulated by a myriad of factors/signaling pathways, including chemo-attractants, like various neurotrophic factors (for instance, ET-1/NGF and NT-3 are critical for sympathetic, whereas GDNF regulates parasympathetic innervation[2]) [38-41] and neural chemorepellents (especially Sema3a[3],

[2] NGF, nerve growth factor; ET-1, endothelin-1; GDNF, glial cell line–derived neurotrophic factor.
[3] Sema3a, Semaphorin 3A.

strongly expressed in the developing heart at E12 in mouse and reduce gradually with development) [42, 43]. Crosstalk and balance between chemo-attractants and chemorepellents guide the proper innervation patterning in the heart.

Dynamic Changes in a Diseased Heart

Cardiovascular diseases (CVD) are the number one cause of death in the developed countries. An estimated 17.9 million people died from CVD in 2016, representing 31% of all global deaths. Of these deaths, 85% are due to heart attack and stroke (WHO 2017). Diminished flow of oxygenated blood to cardiac muscle, such as occurs by occlusion of the coronary arteries, is known as ischemic heart disease (IHD). IHD is the most common form of CVD. According to data published by the WHO in 2020, the number of deaths from IHD increased by more than 2 million since 2000, to nearly 9 million in 2019. IHD can eventually lead to a myocardial infarction (MI) which can further deteriorate outcome, including complications such as sudden cardiac death (SCD), ventricular tachycardia (VT) and heart failure.

Epicardium and Epicardium-derived Cells (EPDCs) in a Diseased Heart. MI induces damage and loss of cardiac tissue, caused especially by the death of cardiomyocytes. The loss of cardiac tissue may lead to loss of cardiac function which in turn can result in inadequate blood supply to the whole body by affecting cardiac output. After cardiac damage, a cascade of events occurs aimed at restoring of cardiac function in the acute phase of MI. One of these events is the (re)activation of epicardial cells.

In the adult mammalian heart the epicardium is, in contrast to the developmental state, a quiescent and single-cell layered tissue with squamous morphology. However, the epicardium is reactivated rapidly after cardiac damage and recapitulates its embryonic epicardial characteristics, including a cuboid morphology [16, 44]. The activation of epicardium begins within a day after MI, reaches its the peak on the fifth day and gradually diminishes after 14 days [45-47]. Typical characteristics of the activated epicardium include: a) the transition of the epicardium from a single-cell layer into a multilayered tissue with cellular expansion and morphology change into a cuboidal shape, as indicated above [44, 47]; b) reactivation of expression of early embryonic epicardial genes, a.o. WT1, Tbx18, TCF21 and Raldh2 (Aldehyde dehydrogenase 1 family, member A2) [48-50].

Activated epicardium (epicardial cells) in the diseased heart thus recapitulates an embryonic phenotype and will undergo EMT, after which the cells are referred to as EPDCs [48, 51-54]. EPDCs can migrate into the subepicardial space and myocardium, where they can differentiate into coronary smooth muscle cells and cardiac fibroblasts in an effort of the heart for self-repair [17, 44, 47, 55]. The reactivated epicardium is also considered to serve as a source of

paracrine signaling to support repair, for example to reduce post-MI infarction size and improve heart function [47, 56, 57]. Remarkably, reactivated epicardium/EPDCs can modulate cellular and paracrine inflammatory process after cardiac damage, which is involved in the recruitment of cardiac macrophages and immunosuppressive regulatory T-cells [58-61].

Cardiac Autonomic Nervous System Changes in a Diseased Heart. An increasing amount of evidence indicates a change of cardiac innervation in cardiac disease states, not only after MI but also in a broad range of other cardiovascular disease, like hypertension, heart failure and pressure overload-induced cardiac hypertrophy [62-64]. These cardiac autonomic nervous changes include alterations in neurochemistry and activity (*functional*) as well as in innervation patterns (*structural*). Alterations of cardiac innervation can disturb the balance between sympathetic versus parasympathetic neuronal activity and may predispose patients lethal arrhythmias [65].

In contrast to the atria, cardiac ventricles are mainly innervated by sympathetic (efferent) nerves and only sparsely innervated by parasympathetic (efferent) nerves. In case of MI, cardiac tissue in the infarction and peri-infarction area will initially undergo functional and structural denervation, caused by death/degeneration of sympathetic fibers and by inflammatory cytokines (for example from the gp130 family) [66, 67]. Inflammatory cytokines can cause local suppression of TH, norepinephrine transporters as well as norepinephrine and can increase TH degradation [68-70]. After the initial denervation, both a functional and structural cardiac reinnervation occur which has been well characterized in animals and humans [71-74]. However, an overshoot of regeneration of sympathetic nerves can also occur, which is designated sympathetic hyperinnervation, defined as an increase of sympathetic fibers in the heart, and has been associated with ventricular arrhythmias and sudden cardiac death [75, 76]. Co-existence of sympathetic denervation and hyperinnervation can also trigger serious ventricular arrhythmias [77, 78]. The exact mechanism of the occurrence of hyperinnervation and it relation to cardiac arrhythmias is not clear yet. Neurotrophic factors released after MI likely play a key role in sympathetic re-/hyperinnervation, particularly NGF, which can be synthesized and released by cardiomyocytes, Schwann cells and inflammatory cells [40, 79-81]. Aside from the alterations of cardiac sympathetic innervation with regard to function and structural patterning, remodeling and transdifferentiation of neuronal cells has been reported [82-84]. These findings expand present mechanistic understanding of the cardiac nervous system in the pathological heart, and further investigations in this field could promisingly yield novel therapeutic targets for heart disease. In this thesis, we aim to further explore this by studying the remodeling of the autonomic nervous system after cardiac damage. We especially focus on the role of EPDCs in cardiac autonomic innervation.

Scope and Outline of the Thesis

Chapter 1 provides the context of this thesis with background information regarding the development of the heart, the critical contribution of the epicardium and its derived cells in cardiac development and the development of the cardiac autonomic nervous system. In addition, the cascade of events after cardiac damage, such as MI is discussed, including the occurrence of cardiac sympathetic hyperinnervation and its clinical sequalae.

Chapter 2 describes how human activated EPDCs can stimulate cardiac sympathetic innervation. We show that EPDCs significantly enhanced sympathetic neurite sprouting directionally towards damaged myocardium *in vitro.*

Chapter 3 demonstrates that sympathetic neurite outgrowth *in vitro*, is influenced by the sex of the donor of EPDCs. The data underlines the potential relevance of sex differences in post-MI cardiac hyperinnervation.

Chapter 4 describes the establishment of a polyclonal line of inducible proliferative human EPDCs (iEPDCs) cells, which was achieved by doxycycline-controlled expression of simian virus 40 large T antigen (LT) with a repressor-based lentiviral Tet-On system. As primary human EPDCs have very limited proliferation ability *in vitro*, iEPDCs are highly useful for *in-vitro* studies that take advantage of EPDCs in the experimental design.

Chapter 5 addresses the neuronal remodeling of murine superior cervical ganglion (SCG) and the proximate carotid body in sequential time point after myocardial infarction. The results demonstrate an overt neuronal remodeling that occurs in the SCG as well as in the carotid body, suggesting an interaction of these 2 structures after MI, that might contribute to pathological cardiac hyperinnervation.

Chapter 6 provides a detailed protocol of low-input nucleus isolation and multiplexing with barcoded antibodies used for single nucleus RNA sequencing (snRNA-seq) of murine SCG. This method enables long-term sample preservation maintaining an adequate RNA quality when samples cannot be fully collected within a short period of time. Moreover, hashtag barcoding antibody-oligos (HTOs) staining enables demultiplexing and the trace-back of distinct ganglionic samples during the subsequent single nucleus analysis.

In **Chapter 7** We aimed to reveal the cellular composition and molecular signature of healthy murine superior cervical ganglia (SCG) by using snRNA-seq. The analysis is focused on the cellular heterogeneity of neurons and satellite glial cells. Analysis of sex and laterality differences of SCG is also included in this chapter. The data provides a useful resource for further disease-oriented studies.

Chapter 8 provides a general discussion on the relevance of the SCG in health as well as in cardiac disease. The discussion focuses on morphological evidence of a contribution of the SCG in cardiac innervation in different animal species including human.

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