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Review article

Wnt Signaling: The double-edged sword diminishing the potential of stem cell therapy in congenital heart disease

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ABSTRACT

Stem cell therapy using bone marrow derived or mesenchymal stem cells has become a popular option for cardiovascular disease treatment, however the administration of embryonic stem cells has been mostly experimental. Remarkably, most of these ongoing clinical trials involve adult patients, but little is known regarding the safety and efficacy of stem cell therapy in newborns and children battling congenital heart diseases. Furthermore, cell delivery methods involve the administration of stem cells without pre-differentiation, and without consideration for the consequent process of cardiac development. Interestingly, *in-vitro* studies have demonstrated that the differentiation of embryonic stem cells into cardiomyocytes imitates the stages of cardiogenesis. Wnt signaling plays a profound role during the earliest stages of cardiogenesis and cardiac differentiation. In fact inappropriate Wnt signaling is associated with numerous cardiac disorders especially congenital heart disease. Furthermore, cell-extracellular matrix interactions were shown to be critical for stem cell differentiation and adequate cardiogenesis. Since extracellular matrix molecules are fundamental for maintenance and repair during heart disease and congenital heart disease, they may offer a novel approach for therapy. Herein we aim to review the critical role of Wnt signaling, as well as the profound importance of cell extracellular matrix interaction, during cardiogenesis. Both of these processes are crucial for precise stem cell differentiation into cardiomyocytes and developing efficacious regenerative therapy for congenital heart disease.

1. Background

Congenital Heart Diseases (CHD) are the most common class of congenital malformations, with an occurrence of approximately 1% in live births, and 10% of aborted fetuses [1]. While surgery remains the gold standard treatment option for CHD, the risk of developing ventricular dysfunction after surgery continues to increase as the child grows older [2]. Many patients endure repeated interventions and multiple corrective surgeries as they grew older, leading to an increased risk of developing heart failure. The current therapeutic option for this final stage of CHD would be heart transplantation, which currently suffers severe donor shortages [2]. Stem cell therapy and regenerative medicine have thus recently served as complimentary and alternative treatment options for CHD patients [3].

Cardiac tissue engineering has recently made rapid advancements in both inschemic and non-ischemic heart disease including CHD [3]. Recent studies showing the higher regenerative potential of newborns and young children compared to those of adults [4] suggest promising outcome for cardiac regeneration therapy and stem cell-based therapeutic strategies in CHD in the pediatric population.

Several ongoing studies on stem cell therapy for CHD indicate that this approach could address the unfulfilled clinical needs of patients with CHD [2,3]. In fact, bone marrow-derived stem cells (BMSCs), bone marrow mononuclear cells (BMMNC), mesenchymal stem cells (MSCs) as well as umbilical cord blood cells have been all administered to treat CHD, although treatment has been limited to single cases and small-size cohorts [5]. Additionally, the quality of the studies was compromised by inadequate control and insufficient follow-up [3]. Nevertheless the administration procedure appeared to be feasible, safe, and without any serious adverse events [6]. Similar to MSCs, the transplantation of allogeneic cardiosphere-derived cells (CDCs) has proven safe and beneficial, and has been used in phase I, II and III clinical trials [5]. As a result of the number of satisfactory outcomes of the clinical trials using stem cell therapy in children with CHD reported between 2010 and 2017 [3], the potential of stem cell therapy in CHD appears to be promising.

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Despite the encouraging results observed in these preliminary studies, data on the safety and effectiveness of stem cell therapies for newborns and children with CHD remains inadequate. For instance, in the majority of the clinical trials, stem cells, regardless of their source, were administered without pre-differentiation, and without consideration for the subsequent cardiac development [3]. Moreover, systematic evaluation of the fate of the administered cells, their integration in cardiac tissue as well as functional efficacy is typically lacking. Since a large proportion of the ongoing studies focus on the development of cell-seeded grafts and patches that are designed to grow in parallel with whole body growth of the infant [3], it is imperative to understand the process of cardiogenesis, in order to mimic embryonic cardiac development when applying stem cell therapies in cases of CHD.

Recently, cardiac progenitor cells (CPCs) derived from human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) have shown great promise both *in vitro* and in pre-clinical trials modeling pulmonary artery hypertension and right ventricle (RV) dysfunction or failure [3,7]. These CPCs are able to populate manifold lineages of the heart and significantly improve myocardial function [8]. However, despite the recent advances in stem cell therapy, subjects still appear to be at risk of exposure to tumorigenicity and developing arrhythmogenicity following the administration of the highly plastic stem cells [3]. Similarly, and in spite of improved embryonic stem cell (ESC) differentiation protocols, their high plasticity makes them difficult to control and their response, once in the recipient, some how difficult to predict [9].

Animal studies demonstrated that the high regenerative capacity of cardiomyocytes during the neonatal period maybe lost during adulthood [4]. To determine whether this dormant regenerative response could be reactivated in adulthood, it is necessary to determine the potential molecular inducers of CPC differentiation during embryonic development of the heart. A better understanding of these pathways would expand the potential of the regenerative capacity of the heart and help create a guide for employing the correct cell type and differentiation cocktails for cardiac regeneration in the pediatric population.

Differentiation strategies employing ESCs and iPSCs have strongly benefitted from translating developmental concepts to *in vitro* culture systems [8]. This suggests that understanding and manipulating these pathways could allow for more efficient generation of cardiomyocytes for future clinical use. In another study on a non-human primate model of myocardial ischemia-reperfusion, cryopreservation and intra-myocardial delivery of 1 billion cardiomyocytes derived from hESC using a differentiation cocktail of Activin A/BMP 4 resulted in significant regeneration of the infarcted heart [10]. A similar positive response was observed but in a large animal model of overloaded RV dysfunction mimicking the repaired tetralogy of Fallot (ToF) [11]. Together, these findings highlight the importance of targeting the signaling pathways that participate in the differentiation of CPCs during the early developmental stage for the generation of cardiomyocytes for cardiac repair applications.

The extracellular matrix (ECM) and its molecular components are a part of a finely orchestrated system for tissue development and repair [12]. Many cellular activities that encompass development, differentiation and survival are mediated by the interaction of cells with their specific ECM environment [13]. For example, matricellular proteins including tenascin C and X, and osteopontin play a vital role in the maintenance, and repair of most tissues [12,13]. Components of the ECM are synthesized and secreted by embryonic cells at the earliest stages of development [12]. During these very early stages the ECM functions to provide a structure for adhesion and facilitating the crosstalk between growth factor receptors and ECM receptors [12]. Moreover, studies in zebrafish demonstrated that the non-collagenous ECM proteins fibronectin and laminin are vital for myocardial precursor migration [12], which appears to be extremely important for formation of the primordial heart tube in zebrafish. Recent studies have also demonstrated that the ECM is critical during both embryonic development and the onset of cardiovascular disease [14]. However, little is known of the *'niche'* microenvironment, or the complex and highly specialized environment, surrounding the developing cardiac lineages of cells [15], more specifically the interaction between CPCs and components of the ECM, and how this communication is impaired in CHD. Understanding the role of the cell-ECM interactions during cardiogenesis could thus translate into more efficacious regenerative therapy for CHD.

2. Development of the bilaminar embryonic disc and primitive streak

The heart is the earliest organ to functionally develop in humans, and begins to form at around the third week of gestation [16]. However, in order for accurate cardiac development to occur, several cellular processes need to be tightly controlled by a well conserved set of molecular pathways [17]. The pathways controlling the differentiation of ESCs into cardiac cells include bone morphogenetic proteins (BMP), fibroblast growth factor (FGF), nodal and Wingless (Wnt) signaling pathways [7]. Embryological development has offered invaluable insight into these key pathways regulating ESC maintenance and tissue homeostasis [9]. The canonical Wnt pathway involves nuclear recruitment of β-catenin through its ligands Wnt1, Wnt3a, and Wnt8, as well as activation of Wnt-dependent transcription factors, while some ligands (Wnt5a or Wnt11) also activate non-canonical cascades such as the Wnt/planar cell polarity (PCP) and the Wnt/Calcium (Ca^{2+}) pathway [18]. The slightest deviation in any of these pathways can result in serious repercussions and has recently been suggested to be the culprit underlying CHD and several ischemic heart diseases [19].

Understanding the differentiation of ESCs to the cardiac lineage as well as the development of the heart during embryogenesis is thus essential for developing *in vitro* protocols for potential stem cell therapy. Morphologic changes occurring in the post-implantation blastocyst create a bilaminar embryonic disc, composed of an epiblast (primitive ectoderm) and hypoblast (primitive endoderm) layer of cells [20]. This structure is arranged so that the epiblast lies adjacent to the amniotic cavity, while the hypoblast cells form the roof of the secondary yolk sac (Fig. 1A) [21]. The layers of both the epiblast and hypoblast are peripherally continuous with extraembryonic tissues on the exterior of the embryo [20]. This extraembryonic structure is essential for intrauterine embryonic development of the placenta and fetal membranes [22].

A grove created in the epiblast allowing cells to migrate inwards, known as the primitive streak, begins to form caudally in the median plane of the dorsal side of the bilaminar embryonic disc, with the primitive node forming at the cephalic end [22]. The appearance of the primitive streak appears to be controlled by canonical Wnt signaling through WNT3a secretion, in cooperation with Nodal signaling [23]. In fact, a feedback loop has been proposed whereby Nodal signals to the extraembryonic ectoderm activates BMP 4 expression [24]. Consequently BMP 4 signals back to the epiblast to activate Wnt signaling in the primitive streak [24]. While the streak arises from the epiblast, the orientation of the primitive streak is regulated by the rotation of the hypoblast, where mediolateral cell intercalation at the embryonic epiblast causes narrowing and midline elongation of these cells, which contains the majority of streak precursors [23]. This process requires non-canonical Wnt/PCP signaling (expression of flamingo, prickle and Vang-like 2 factors) and temporary Nodal and *epithelial-mesenchymal transition* (EMT) blockage [23].

The primitive streak usually diminishes in size and eventually disappears after undergoing progressive transformations. However, remnants of the streak may persist and give rise to a sacrococcygeal teratoma (SCT), the most common congenital tumors in neonates, reported in approximately 1/35 000 to live births [25]. SCTs contain tissues derived from the ectoderm, mesoderm, and endoderm, and are believed to arise early during gestation from the totipotential cells of the primitive knot [26]. Although the great majority of SCTs are benign at birth, malignant transformation can occur in children as they

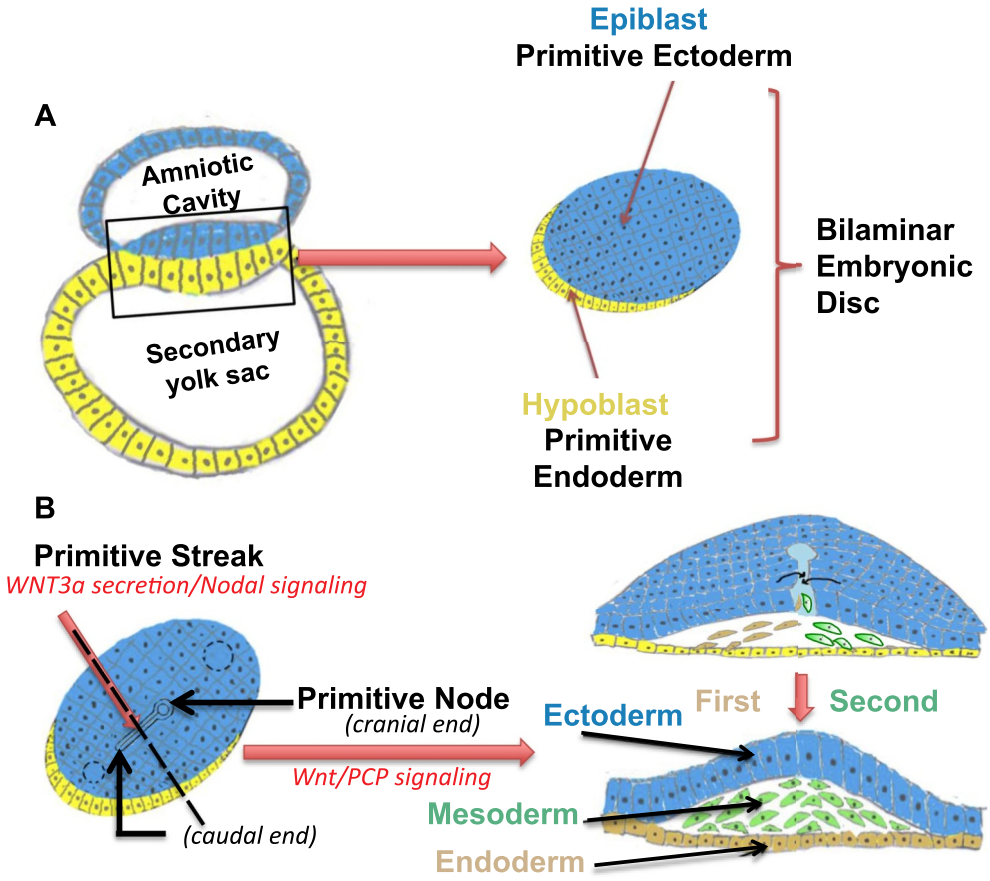

Fig. 1. Gastrulation, mass-cell movement resulting in the formation of the three germ layers of the embryo. A. The fluid-filled amniotic cavity forms the innermost fetal membrane, while the secondary yolk sac is the first extraembryonic structure to develop. Morphologic changes occurring in the post-implantation blastocyst create a bilaminar embryonic disc, composed of an epiblast (*primitive ectoderm*) and hypoblast (*primitive endoderm)*. **B.** The primitive streak is a grove created in the epiblast, which begins to form caudally in the median plane of the dorsal side of the bilaminar embryonic disc, with the primitive node forming at the cephalic end. The appearance of the streak is governed by canonical Wnt signaling through WNT3a secretion, in cooperation with Nodal signaling, while the orientation of the primitive streak requires non-canonical Wnt/PCP signaling and transient Nodal blockage. Formation of the primitive streak allows cells from the epiblast to migrate inwards into the region of the node and streak via an epithelial-mesenchymal transition. This transition forms the three germ layers of the embryo; ectoderm, mesoderm and endoderm.

advance with age [25]. The most commonly produced tumor marker of a malignant SCT is the alpha fetoprotein (AFP) [26], which function has recently been associated with activation of Wnt signaling and promotion of β-catenin-mediated transcription factors (TCF1/TCF7) activity [27]. This recent finding supports that even slight Wnt signaling alteration could significantly impact manifesting congenital birth defects, thereby shedding light on the importance of Wnt signaling in pathogenesis, and therapeutic approaches to these defects.

3. Gastrulation and the formation of a three germ-layered embryo

Gastrulation, the tightly controlled process of mass cell movement resulting in the formation of the three germ layers of the embryo; ectoderm, mesoderm and endoderm, is essential for the subsequent development of major body organs, including the heart [28]. The expression of different levels of the agonists of these pathways, combined with region-specific expression of inhibitors, appears to orchestrate signaling domains that regulate germ layer induction and specification in the mouse embryo [9]. Moreover, morphogenetic studies in mice, chick and zebrafish [23,29] demonstrated that in order for epiblast cells to fold at the site of primitive streak formation and subsequent cell migration and EMT shift to occur, cells in the streak must downregulate the expression of genes encoding for ECM components [24]. Embryological studies have demonstrated that gastrulation is a multifaceted process, which is controlled, in part, by the coordinated stimulation and regional inhibition of the Wnt, Nodal, and BMP-signaling pathways [24], as well as interventions from the ECM.

The first morphological evidence of the process of gastrulation is the appearance of the primitive streak. The next step involves the migration of cells from the epiblast into the region surrounding the primitive node and streak via EMT [19]. This process is regulated by multiple overlapping pathways including Notch and transforming growth factor β (TGFβ), which is an activator of canonical Wnt signaling, as indicated by translocation of β -catenin to the nucleus where it acts as a transcription factor [30]. The process of EMT forms mesenchymal stem cells (MSCs), a multipotent line of stromal cells that can differentiate into various cell types (Fig. 1B). MSCs can be isolated from a variety of tissues, including bone marrow, umbilical cord, and adipose tissue [31]. *In vitro*, MSCs have been shown to differentiate into both cardiomyocytes as well as vascular endothelial cells [31], suggesting an important role for them in enhanced neovascularization and cardiac protection.

Matricellular proteins, of the ECM including tenascin C and X, as well as osteopontin, are also associated with both morphogenesis and vascular growth [13], indicating that communication between cells and the ECM is also key during gastrulation and development. Recent studies have shown that interventions in ECM components have significantly altered the regulation of cell behavior during gastrulation, including providing cues for cell adhesion and migration [24]. Interestingly, engrafting MSC-loaded “cardiac cell patches” has been shown to promote angiogenesis and repair of the infarcted myocardium in mice [32]. In mice subjected to transaortic constriction pressure overload, Wnt/β-catenin signaling contributed to TGFβ-1-induced cardiac fibroblast activation and fibrotic ECM gene induction, during fibrotic remodeling of the heart [33]. These data suggest that reactivation of Wnt is achieved in many heart pathologies. Maintaining a tight control over the manipulation of Wnt signaling and the ECM components may thus significantly impact the potential safely and effectiveness of engrafting stem cell patches in CHD. Unfortunately the major setback of the small size and cell death of the graft tissue currently limits the benefit of this therapy, and requires further investigation.

Recent studies have suggested that regulated cell adhesion mediated by the canonical Wnt/β-catenin pathway and actin cytoskeleton linking [34] is one of the driving forces of morphogenesis during gastrulation [35]. *In vitro*, the expression of cadherin (a key regulator of cell-cell

adhesion) was directly correlated with the ability of reconstituted embryonic cells to resolve to the different germ layers and to arrange into their proper anatomical positions [34]. Cell migration has been interestingly linked to the non-canonical Wnt/JNK and Wnt/Ca^{2+} pathways as a result of cytoskeletal rearrangement [36]. These recent advances have highlighted the importance of the interaction between the migrating cells and the surrounding environment. These dynamic interactions, mediated by adhesion molecules, are fundamental to the effectiveness of stem cells administered for therapy of damaged cardiac cells. Understanding the interaction of the transplanted cells and their host microenvironment, especially the ECM, basement membrane material, and other niche factors is essential for optimizing stem cell therapy.

Following EMT, MSC detachment from the ectoderm occurs in two waves as they ingress at the primitive streak in the space between the transforming epiblast and the hypoblast (Fig. 1B) [22]. The first wave of cells replace the hypoblast to form the definitive endoderm, a process that involves evolutionary conserved signaling pathways including Wnt signaling [24]. The second wave of cells form the mesoderm layer between the epiblast (now referred to as the ectoderm) and the endoderm [28]. Previous studies have shown that the formation of the mesoderm, a source of CPCs, from the pluripotent epiblast depends upon canonical Wnt/β-catenin signaling [37]. In fact, studies have shown that mice devoid of the canonical ligand Wnt3 or β-catenin fail to develop a mesoderm, resulting in defective anterior-posterior axis formation [38]. These findings suggest a necessary requirement for canonical Wnt/β-catenin signaling in the earlier stages of development (Table 1).

The formation of the mesoderm activates expression of the earliest transcription factors required for development of the cardiac crescent, *Mesp1* and *Mesp2* in the primitive streak [19,36]. Following the formation of the neural folds, epiblast cells moving through the primitive streak further divide the mesoderm layer into paraxial mesoderm, intermediate mesoderm, and lateral plate mesoderm (Fig. 2) [20]. Fibronectin deposited on the basal surface of the lateral plate mesoderm is essential for the proper formation of the cardiac tube [12]. In fact, loss of fibronectin expression in zebrafish during myocardial precursor migration was directly linked to the occurrence of cardia bifida [12], further highlighting a crucial role for cell-ECM interaction during cardiogenesis. As the transformation of the mesoderm is occurring, the intra-embryonic cavity takes shape in the form of a single space located in the lateral plate mesoderm [22]. This coelom would later form the three major body cavities: the pleural, peritoneal and pericardial cavities.

Cells remaining from the epiblast form two midline structures; the prechordal plate and the notochordal process [20]. While the notochord is a mesodermal structure that determines the long axis of the embryo, the prechordal plate, the site of the future mouth, is formed by direct contact between ectoderm the endoderm and is devoid of any mesoderm [21]. The appearance of the notochord and prechordal plate consequently induces the superimposing ectoderm to thicken and form the neural plate, initiating neurulation [22]. Neurulation is vital for the subsequent development of the central nervous system as well as formation of cardiac lineage of neural crest cells [20], sometimes referred to as the fourth germ [22]. Neural crest cells detaching from the lateral margins of the neural folds, undergo EMT and exit the neuroectoderm to enter the underlying mesoderm [22]. The mechanisms that regulate neural crest cell development and migration are currently being further examined, however studies have heavily implicated Wnt signaling pathways [39]. In addition to the general induction of neural crest cells, Wnt genes encode secreted glycoproteins that play critical roles during the development of the OFT, and the heart as discussed below.

4. The role of Wnt Signaling in cardiogenesis

The formation of the heart is a multifaceted process, initiated by the specification of CPCs from the mesoderm, followed by formation of a linear heart tube and subsequently maturation of the heart. As a mesoderm-derived organ, the heart is a potential target for stem cell-based therapy, and so there is great interest in understanding the pathways that regulate process of cardiogenesis and heart development. As discussed above, the canonical Wnt/β-catenin signal transduction pathway appears to be heavily involved in early development. Subsequently, following the specification of the mesoderm, the canonical Wnt pathway is quickly silenced by Wnt antagonists (BMP 2/4, FGF2/4/8 and Shh), canonical Wnt inhibitors (Dickkopf-1 homolog, Dkk1), as well as non-canonical Wnts (Wnt5a, Wnt11), in order to downregulate pluripotency and promote the formation of cardiac mesodermal cells and cardiac progenitor cell specification [40]. Induction of this cardiogenic program is followed by the expression of numerous transcription factors including cardiac markers NK2 transcription factor related locus 5 (Nkx2-5), GATA4/5/6, myocyte enhancer factor 2C (MEF2C), Tbx5/20, heart and neural crest derivatives expressed transcripts (Hand1/2), and Islet Lim homeobox 1 as well as their downstream targets [7]. This complex network of fundamental and developmental transcription factors collaborate to eventually give rise to the primitive form of the heart [40].

The slightest alteration in the expression of Wnt-activated cardiac markers has recently been associated with several congenital heart defects *in vivo* and in humans [41,42,73], Table 1. Nkx2-5 is thought to be a key transcription factor in cardiac looping and in ventricular marker expression, and its mutation gives rise to both hypoplastic right ventricle syndrome (HRVS) and hypoplastic left heart syndrome (HLHS) in humans [7,43,44], Table 1. Furthermore, the transcription factor MEF2C and Hand1/2 both play important roles in the differentiation of the cardiac mesoderm as well as cardiac looping [7]. In fact, any interruption in MEF2C function has been shown to result in downregulation of Hand1/2 and failure in the development of the right ventricle as well as altered cardiac differentiation [7]. Moreover, a recent study showed that a consecutive cohort of 200 unrelated patients suffering from CHD screened positive to MEF2C loss-of-function, and this likely increased their vulnerability to the disease [45]. Tbx5 has also been implicated in all aspects of cardiogenesis in addition to both spatial and temporal expression changes in the cardiac progenitor cell subpopulations [40]. In fact, the mis-expression of limb and heart development transcription factor (Lbh) has been shown to interfere with non-canonical Wnt signaling through Tbx5 pathway molecules, resulting in right ventricular hypoplasia and pulmonary valve defects [44], Table 1. Collectively, these data could justify why the CPC approach for cell therapy in CHD remains limited, since a tight control is necessary to regulate the expression of these key factors. Accordingly a clearer picture of the signals that control cardiomyocyte differentiation and progenitors for cardiovascular cells need to be better defined.

Multipotent CPCs migrate after their ingression through the primitive streak towards the splanchnic part of lateral plate mesoderm giving the primary heart field (PHF) and the secondary heart field (SHF) [21] (Fig. 2). This process appears to be highly regulated by Wnt2 through the canonical β-catenin pathway [37]. The splanchnic part of the lateral mesoderm migrates out of the mesodermal layer towards the endoderm to form the endocardial tubes on either side [36]. At the same time the endoderm begins to fold inward, and continues folding inward until it forms its own tube, dragging the two endocardial primordia close to each other [21]. This allows the mesoderm to meet and merge, forming the heart primordium [46]. This process of lateral folding of the embryo brings the heart tubes into the ventral midline.

Craniocaudal folding brings the heart primordium and pericardial cavity, which lie in front of the oropharyngeal membrane (future mouth) and the neural plate, into the thoracic region (Fig. 2) [21]. The septum transversum forms cranial to the cardiogenic area in the developing embryo, and is translocated to the future lower thoracic region through the folding of the cranial end of the embryo [47]. This mesodermal structure divides the intra-embryonic coelom into thoracic and abdominal cavities. Due to the growth of the brain and cranial folding,

Table 1
The role of Wnt Signaling in Gastrulation, Heart Development and Congenital Heart Disease (CHD). A summary of the role of canonical Wnt/β-catenin and non-canonical Wnt signaling in early gastrulation events, cardiomyocyte differentiation, cardiogenesis and subsequent development of the heart. Several CHDs in which aberrant Wnt signaling has been implicated are shown (studies in blue text (*in vivo*), in red text (*in humans),* N/R; *not reported*).

Process	Role of Wnt in Process	Role in Congenital Heart Disease
Appearance of Primitive Streak	Canonical Wnt signaling through *Wnt3a*	N/R
Epithelial to Mesenchymal transition	Canonical Wnt/ -catenin signaling	N/R
Formation of the definitive Endoderm	Canonical Wnt/ -catenin signaling	N/R
Formation of the Mesoderm	Canonical Wnt/ -catenin signaling	N/R
Formation of multipotent Cardiovascular Progenitor Cells (CPC)	Non-canonical Wnt signaling through *Wnt5a* and *Wnt11*	Loss of *Wnt5a* leads to septation defects of the cardiac outflow tract (OFT) in the form of **Persistent Truncus Arteriosus (PTA)** (Schleiffarth et al., 2007) *Wnt11* null mice revealed **Ventricular Septal Defects (VSD)** and malformations leading to **Double Outlet Right Ventricle (DORV)** (van Vliet *et al.*, 2017) **VSD** is associated with lower expression of *Wnt11* in infants In **Tetralogy of Fallot** infants, downregulation of *Wnt11* expression was associated with lower oxygen saturation (Touma et al., 2017)
Migration of CPC to Primary Heart Field (PHF) and Secondary Heart Field (SHF)	Canonical Wnt signaling through *Wnt2*	Suppressed canonical Wnt signaling causes fibro-adipocytic replacement of cardiomyocytes in the epicardium resulting in **Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC)** (Lombardi and Marian, 2011) Dysregulation of the stem cell extracellular niche could also be key for the phenotype of **ARVC** observed (Lombardi and Marian, 2010)
Formation of the linear cardiac tube	Non-canonical Wnt/Ca^{2+} signaling through *Wnt11*	N/R
Cardiac looping and Ventricular marker expression	Non-canonical Wnt signaling	*Limb and Heart development transcription factor (Lbh)* mis-expression interferes with non-canonical Wnt signaling through *Nkx2-1/Tbx5* pathway molecules, resulting in **Hypoplastic Right Ventricle Syndrome (HRVS)** and **pulmonary valve defects** (Dimopoulos *et al.*, 2017) Mutations in *Hand1/Nkx2-5/Notch* result in **Hypoplastic Left Heart Syndrome (HLHS)** (Kobayashi *et al.*, 2014)
Trabeculation and Compaction of the ventricles	Non-canonical Wnt/PCP signaling pathway members	Mice deficient in *Vang-like 2* developed **ventricular non-compaction** (Phillips et al., 2007)
Valvulogenesis	Canonical Wnt/ -catenin signaling	Disruption Wnt/ -catenin signaling during valvulogenesis could contribute to **Bicuspid Aortic Valve** (BAV) (Combs and Yutzey, 2009)
Outflow Tact (OFT) formation	Canonical Wnt/ -catenin signaling target genes	Repression of *Protocadherin 4/C-Terminal Binding Protein 1* gene expression results in aberrant Wnt signaling, resulting in OFT malformations. **Overriding Aorta** is a pathological feature of **TOF** (Liu *et al.*, 2017)
	Non-canonical Wnt/PCP signaling members	Mutations of *Vang-like 2/Dishevelled* results in abnormalities in the remodeling of the OFT, resulting in **DORV** (Phillips et al., 2005)

the heart and the pericardial part of the intraembryonic coelom move to the thoracic region [21]. As the embryo continues to develop, the ectoderm and amniotic membrane undergo rapid growth so that the entire surface of the embryo is covered with ectoderm (except at the umbilicus), and the amnion surrounds the entire embryo [47]. Not only does the amniotic membrane provide the developing embryo with support and protection, it has recently been identified as a source of stem cells, with numerous potentials for therapy [21].

Traditionally, it was believed that all cardiomyocytes simply originated from the cardiac crescent [17], which was considered to be the PHF [36]. This notion was quickly overturned at the discovery that cells from the SHF contributing primarily to the OFT region of the heart as well as the right ventricle, while cells from the PHF contribute primarily to the left ventricle (reviewed in Refs. [17,36,40]). Furthermore, retrospective clonal analysis suggests that both lineages differ with respect to the onset of terminal differentiation with cells from the SHF terminally differentiating later than those from the PHF [36]. Thus the PHF is distinguished from SHF and acknowledged as a distinct structure. Both the craniocaudal and lateral folding become even more important during development, as the they transform the developing

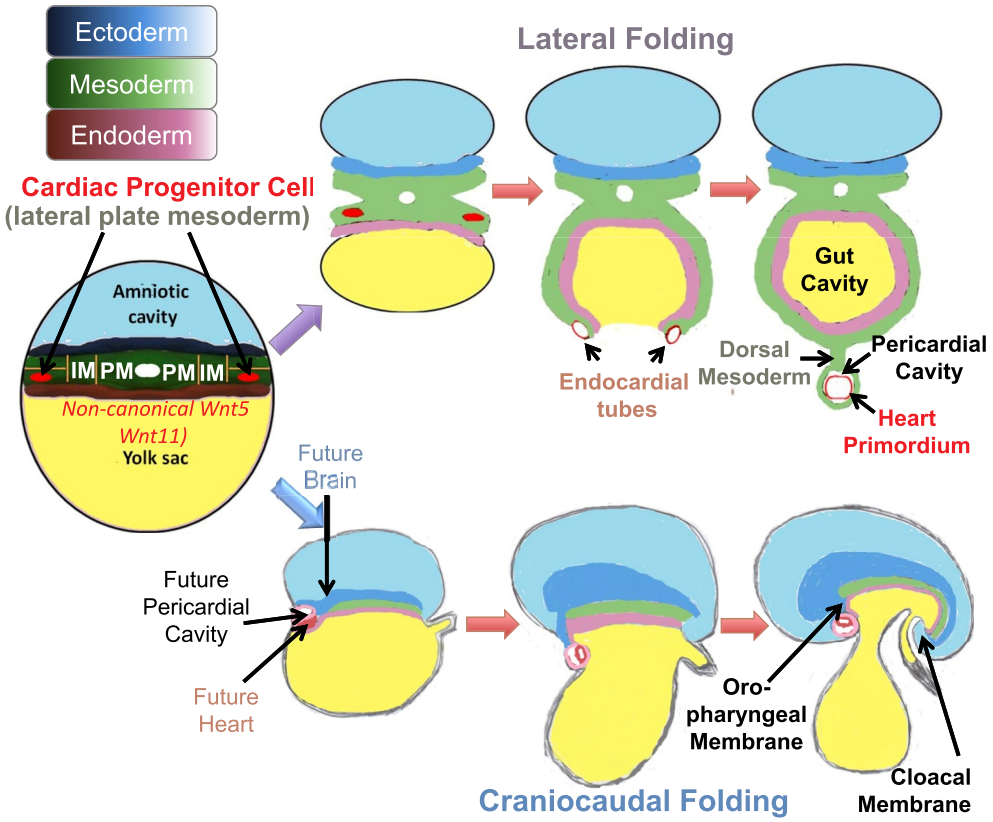

Fig. 2. Division of Mesoderm and Lateral-Craniocaudal folding that transforms the embryo into a three-dimensional vertebrate body. Epiblast cells moving through the primitive streak further divide the mesoderm into paraxial mesoderm (PM), intermediate mesoderm (IM), and lateral plate mesoderm, where the cardiac progenitor cells (CPC) form, a process governed by the silencing of canonical Wnt by Wnt antagonists and non-canonical Wnts (Wnt5a, Wnt11). The cardiogenic mesoderm migrates out of the mesodermal layer towards the endoderm to form endocardial tubes on either side, when the endocardial tubes get close enough; they fuse together and form the heart primordium. As craniocaudal folding occurs, the embryo grows more rapidly than the yolk sac bringing the heart primordium and pericardial cavity into the thoracic region, while the cranial rim of the embryo contains the oropharyngeal membrane and the caudal end contains the cloacal membrane.

embryo from a flat disc into a three-dimensional vertebrate body [21]. Any alteration in this order of events would hinder the formation of the primordial heart and realignment of the embryo. The subsequent successful conjunction of right and left pre-cardiac regions in the anterior ventral midline of the developing embryo results in the formation of a linear heart tube [40], also known as the cardiac tube (Fig. 3).

The primitive form of the heart is composed of an inner layer of endocardial cells and an outer layer of myocardial cells and becomes functional by week 3–4 in humans [7]. In fact, the heart beat starts spontaneously as myocardial cells express the sodium-calcium pump, even before fusion is complete [48]. These early heartbeats help guide the growth of cardiomyocytes and shape the developing heart [48], however, the exact mechanism remains unclear. Since Wnt11 expression is required for heart specification as well as for promoting cardiogenesis, it is possible that this process results through activation of the non-canonical Wnt/Ca^{2+} pathway (Fig. 3). Given the important role that Wnt signaling plays in both cardiomyocyte differentiation and their stem cell origin, in addition to tissue regeneration in general, understanding this pathway is important to improve our knowledge of both cardiovascular development and cardiac reparative mechanisms.

5. The role of Wnt Signaling in the development of the heart

In order for the heart to continue to mature, it is necessary for the linear cardiac tube to expand. This is accomplished via the differentiation and proliferation of cells in the PHF and the addition of cells from the SHF, to the arterial and venous poles of the cardiac tube [19]. These two processes have also been shown to be heavily regulated by both the canonical and non-canonical Wnt pathways (Fig. 3) [36]. Both the PHF and SHF contain a population of epithelial cells that express the key cardiac developmental transcription factors discussed earlier [49]. These include members of the BMP family, namely BMP 4, FGF, Activin A, nodal, TGFβ and Notch proteins, which collaborate in a series of intricate positive and negative feedback loops to induce cardiogenesis [7]. The cardiac tube will then differentiate into the truncus arteriosus, bulbus cordis, primitive ventricle, primitive atrium, and the sinus venosus by undergoing a series of looping so that the rough trabeculated part of the primordial ventricle becomes mainly derived from left ventricle, and the bulbus cordis gives rise to most of the right ventricle [50]. The process of looping and subsequent expansion and maturation of the heart is also tightly regulated by Wnt signaling and its downstream effectors [17].

The process of heart development is a multifaceted process that involves intricate steps such as cardiac tube formation, cardiac looping and chamber formation [50]. During cardiac morphogenesis, looping of the cardiac tube is followed by the formation of the two atria and ventricles of the heart, a process that requires alignment of the OFT and realignment of the cardiac chambers [50], as well as remodeling of the inner heart curvature [7]. Growth of the ventricular wall is complemented by the development of distinct trabeculated and compact myocardial layers. The latter is important for cardiac chamber wall thickening and septation, force generation and valvulogenesis [40]. Tight control of the ECM dynamics along with Notch and Neuregulin signaling from endocardium are crucial for the development of the trabecular construction scheme during cardiac chamber formation [51]. Delayed termination of the Notch pathway has been identified as a causative factor for non-compaction cardiomyopathy, a rare CHD characterized by excessive trabeculation and defects in the maturation of the ventricular wall [51]. Interestingly, mutant mouse models lacking key Wnt/PCP signaling components such as Vang-like 2 also appear to develop ventricular non-compaction, suggesting that non-canonical Wnt/PCP signaling is a key pathway in cardiomyocyte polarization and ventricular compaction [52], Table 1. Whether there is interplay between aberrant Wnt and Notch signaling in non-compaction cardiomyopathy is yet to be determined. However, these findings highlight a clear and emerging role of Wnt signaling in CHD.

The next process in cardiac development involves chamber septation and the formation of the interatrial septum, which segregates both

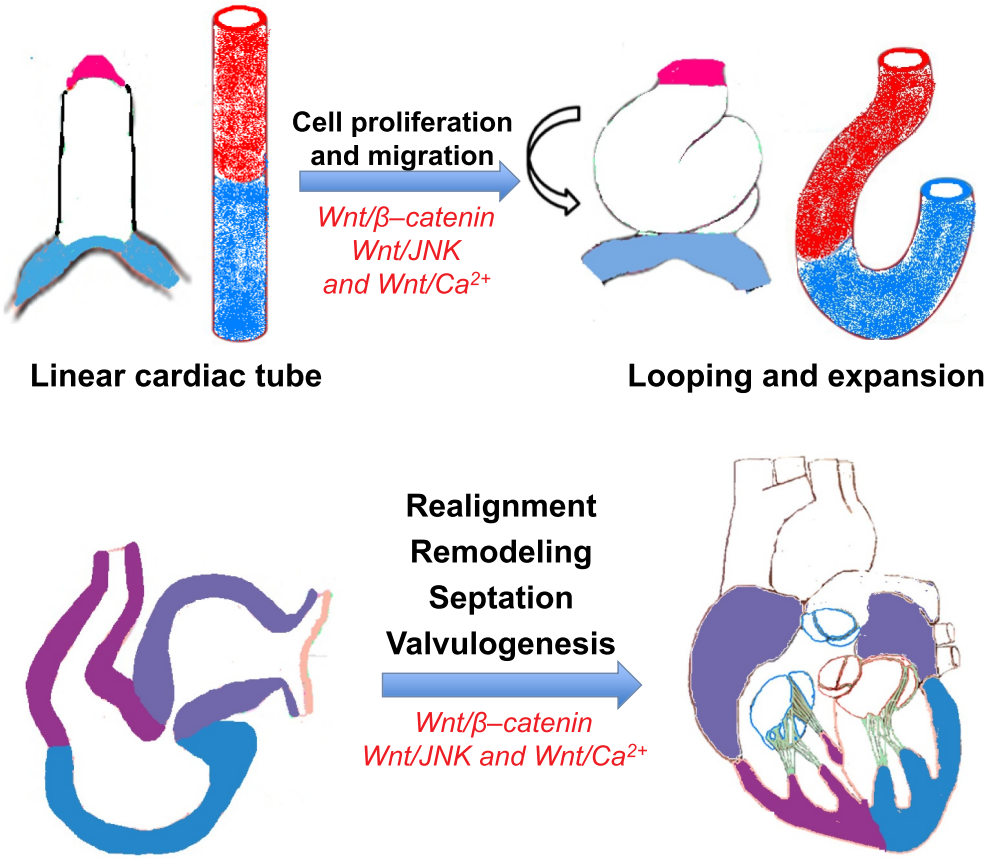

Fig. 3. Schematic representation of the dynamic role of Wnt signaling during cardiogenesis. Both canonical and non-canonical Wnt signaling are crucial for cell proliferation and migration early during the formation of the linear cardiac tube, and subsequent looping and expansion. The process of alignment and realignment, subsequent septation, remodeling and valve formation of the cardiac tissue is also tightly controlled by Wnt signaling.

atria, as well as the interventricular septum (IVS), which divides the right from the left ventricle [50]. To our knowledge, no data is currently available on the function of Wnt signaling during chamber septation, although in Wnt2b knockout embryos, the primary atrial septum (a crescentic muscular membrane descending from the roof of the atrium during formation of the interatrial septum), does not form [36]. The first sign of valvulogenesis in the developing heart is the establishment of endocardial cushions (EC) in the atrioventricular canal and OFT of the looped cardiac tube [53]. MSCs derived by the EMT of overlying endocardial cells mobilize to delaminate and occupy the ECM of the cushions, rapidly after they form [53]. At this stage, the ECM is referred to as cardiac jelly, which plays an important role in regulating cell migration and proliferation in the developing heart [36].

During valvulogenesis, the canonical Wnt pathway appears to have important functions [37]. Data from Wnt reporter transgenic mice imply that canonical Wnt signaling is active in the atrioventricular canal and OFT cushion mesenchyme during the growth phase that follows EMT and cushion expansion [53]. Moreover, overexpression of Wnt inhibitors in zebrafish blocks the formation of EC in the atrioventricular canal [36]. As the heart continues to develop, the valve primordia formed by the EC become longer, turning into thin cusps (which later develop into aortic and pulmonic semilunar valves), or leaflets (which makeup the atrioventricular valves) [36]. In spite of morphogenetic and structural differences between atrioventricular and semilunar valves, the molecular mechanisms of valve development are relatively conserved between their valve leaflets [30]. During the late stages of gestation and postnatal period, the valve leaflets become stratified into highly structured collagen-proteoglycan-elastin-rich ECM compartments [30]. Interestingly, the underlying abnormalities of many severe congenital valve defects that require childhood intervention originate from disruption of embryonic valvulogenesis [53]. Disruption of the aforementioned Wnt/β-catenin signaling roles, which enable valvulogenesis, could account for common congenital valve defects such as bicuspid aortic valve (BAV) [30], Table 1.

The proper development of the cardiac neural crest cells is essential for both the development of the OFT [36] and the establishment of cardiac functions [54]. Defects during the migration of the cardiac neural crest result in congenital heart defects such as persistent truncus arteriosus (PTA), in which loss of the non-canonical Wnt5a ligand leads to OFT septation defects [55], Table 1. Other congenital heart defects are considered neural crest defects as well, and specifically result in malformations of the OFT. That defect has been reported to be directly linked to aberrant Wnt signaling [56,57], Table 1. For instance, repression of Wnt/β –catenin target gene expression (Protocadherin β 4/ and C-Terminal Binding Protein 1) was shown to induce OFT malformations. Examples include overriding aorta, which is also a pathological feature of ToF [57], Table 1. Moreover, examination of Scrib (another key Wnt/PCP molecule) mutant mice demonstrated altered ventricular abnormalities in the polarization and organization of cardiomyocyte in the outflow myocardium. This alteration gave rise to OFT defects such as Double Outlet Right Ventricle (DORV) [52], Table 1. Wnt 11 has also been shown to regulated OFT development, and ablation of Wnt 11 has also been linked to DORV as well as ToF [73,74]. These results strongly support the hypothesis that both canonical and non-canonical Wnt signaling are essential for the development of the heart and any alterations in either will have dire consequences. Together, these findings call for the evaluation of current stem cell therapeutic interventions, which do not take into account the relevance of Wnt signaling in cardiac regeneration. This understanding may also provides technical clues to improve the current protocols for directed differentiation of ESCs into cells of cardiac lineages, and provide new approaches for the development of novel therapeutic approaches to target CHD.

6. Crosstalk between Wnt and retinoic acid signaling

Wnt signaling appears to be a focal point of an integrated signaling network required for ESC differentiation and CPC expansion in the heart. However, the interplay between multiple signaling systems is also critical for specification and patterning of the heart. This model has been demonstrated in BMP, nodal, Notch and TGFβ signaling pathways [7]. Retinoic acid (RA) signaling has several functions during cardiac development, which include the formation of cardiac progenitors in the lateral plate mesoderm, as well as the correct modeling of the early heart fields [58]. Studies in zebrafish on the other hand indicate that inhibited RA signaling in the lateral mesoderm causes the uncommitted cells to differentiate into myocardial progenitor cells instead of pharyngeal or pancreatic cells. This specialized differentiation leads to the expansion of cardiomyocytes [58]. In the RA-deficient mouse embryos, the anterior part of the SHF was disorganized and posteriorly expanded [59]. This suggests that RA signaling has a dual action during cardiogenesis and the development of the heart, similar to that of Wnt signaling. Interestingly, *in vivo* studies have demonstrated that both the FGF and the Wnt pathways require RA nuclear receptors (RXRα) activation [58,60]. In addition, the absence of the heterodimerization partner of RXRα produced myocardial hypoplasia, suggesting an action for RA on the fetal epicardium that is necessary for myocardial growth [60]. In another study, the Wnt-FGF pathway was found to be downstream of epicardial RXRα. The expression of *Wnt9b*, which encodes a canonical Wnt ligand, was significantly reduced in epicardial-restricted RXRα mutant mice [61]. This was accompanied with thinning in the myocardial wall as well as failure of ventricular compaction. Together these findings further support a crucial role for RA signaling, mediated in part through canonical Wnt signaling, in myocardial growth. The requirement of RXRα signaling in the epicardium for proper cardiac morphogenesis suggests that its regulation may contribute to the beneficial outcomes of stem cell therapy in CHD.

7. Current prospective and future outlook

It is becoming abundantly clear that the canonical Wnt/β –catenin pathway has a biphasic role, in that it induces cardiac specification during early heart development, but inhibits it at later stages [42,62], summarized in Table 1. Additionally, Wnt proteins that act as extracellular growth factors and induce a variety of intracellular signaling pathways have also been shown to play numerous roles during cardiac differentiation and development [36]. Interestingly, manipulation of the appropriate Wnt pathway in zebrafish was recently shown to be essential for the success of cardiac stem cell differentiation and proliferation [63]. Moreover, it has also been demonstrated that early treatment of differentiating mouse ESCs with Wnt3a stimulates mesoderm specification, which activates the feedback BMP 4 loop that subsequently represses the Wnt pathway, thereby increasing cardiac differentiation [29]. In fact the approach of fine-tuning Wnt/BMP 4 pathways in the presence of Activin A was recently use to successfully generate cardiomyocytes from hESC [62]. Collectively these findings further emphasize the importance of maximizing efforts to better understand how to finely manipulate Wnt signaling to efficiently produce cardiomyocytes from ESC or iPSCs for basic studies and potential cardiac repair applications in CHD.

In order to evaluate the safety and efficacy of stem cell transplantation in single ventricle pathologies, preclinical studies have focused on reproducing common HLHS or ToF features including increased pressure or volume overload of the RV in animal models [3]. Intramyocardial injection of CPCs derived from HUES-24 (a hESC line differentiated using a cocktail of both Wnt3a and BMP 2) in the porcine model of RV dysfunction, a feature observed in patients with a repaired ToF before decompensated RV failure, was well tolerated, feasible and showed an improvement in RV tissue remodeling as well as arrhythmic susceptibility [11]. This demonstrates that careful control of canonical Wnt signaling could prove beneficial for adequate stem cell therapy applications in CHD. In spite of these findings, a significant improvement in RV function was not fully achievable. Furthermore, no human cells were detected within the myocardium indicating that the observed improvement in cardiac functions may have been attributed to paracrine effects, not successful differentiation of the CPCs.

Components of the ECM were also shown to interact with, and affect the differentiation of CPCs into the cardiac phenotype [15]. In fact, the relevance of the ECM has been extended to show that its components can provide the natural cues required for optimal cell function [64]. Data from such studies indicate that a naturally derived cardiac ECM could enhance CPC function *in vitro*, in addition to significantly improve proliferation and increase resistance to apoptosis [64]. It is also well established that the ECM stiffness plays a crucial role in controlling the behavior of many cell types; including proliferation, differentiation, spatial organization, and migration [65]. Cells, it seems, can tune their responses to stimuli according to the physical properties of their environment, so much so that it may appear that signals from the ECM can guide stem cells to repair damaged tissues (Gattazzo). Recent data has suggested that Wnt signaling is responsive to ECM stiffness and that the stiffer the ECM, the more β-catenin accumulates as a result of integrin induction [66]. The exact mechanisms of this enhancement remain to be determined, however, this data suggests that integrin-induced-activation of the Wnt/β-catenin pathway could be important in regulating the cell's phenotype and behavior, which could have many implications in regenerative therapy.

To date, only a limited number of clinical trials have assessed the feasibility and effectiveness of stem therapy in pediatric patients with CHD, specifically single ventricle physiology such as HLHS (reviewed in Refs. [2,3,5,6,67]. In the initial study, Transcoronary Infusion of Cardiac Progenitor Cells (TICAP trial) evidence of the feasibility and safety of intracoronary infusion of autologous CDCs in children with HLHS was demonstrated [6]. The outcomes were promising and there appeared to be no complications or tumorigenicity detected at the 3-year follow-up [2]. However this trial had several limitations including the samll study size, nonrandomizion, and that cardiac interventionists were not blinded. Moreover, the majority of the 14 patients enrolled in the trial had undergone the Fontan procedure, stage III of surgical repair for HLHS, during the follow-up period, a procedure that could have easily affected outcome measures [68].

Based on these findings, the phase II randomized controlled Cardiac Progenitor Cell infusion to treat Univentricular Heart Disease (PERSEUS trial) demonstrated that cardiac function in the form of reduced ventricular volumes, fibrosis, somatic growth and heart failure status favorably benefited from the intracoronary delivery of autologous CDCs to 34 patients with single ventricle CHD [3]. Similar to the TICAP trial, CDCs were administered at the same time as stage II or III surgical repair for HLHS; hence it is difficult to determine whether this could have served as a confounding factor. However, it may still be too early to address this limitation, since it would most likely be even more difficult to test the efficacy and safety of such novel cell-based therapeutics if administered at an earlier stage, even if the myocardium is most plastic in earlier childhood. Based on these limitations, it is crucial to focus more global efforts on preclinical studies in which a deeper understanding of the developing embryonic heart and the pathways and factors involved could be translated to the clinical setting at an earlier stage of CHD presentation.

8. Lessons learned from Arrhythmogenic Right Ventricular Cardiomyopathy

The Wnt signaling pathway is also crucial for cardiac tissue homeostasis [42]. The slightest dysregulation in Wnt signaling can result in severe consequences as observed in Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC), an inherited primary disease of the myocardium whose findings commonly overlap with CHD [69]. In a recent

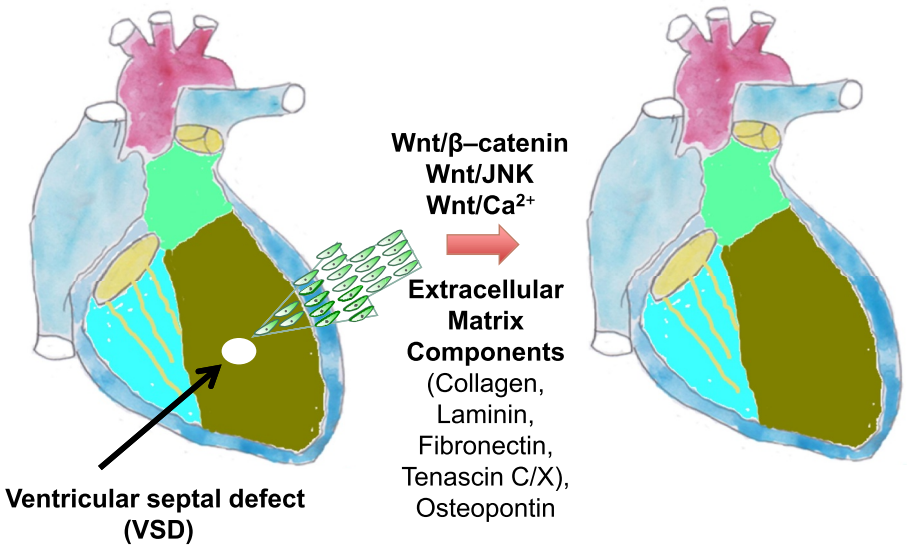

Fig. 4. The importance of Wnt Signaling and cardiomyocyte-extracellular matrix interaction in stem cell therapy. In order to clinically apply the correct cell type for heart regeneration in congenital heart diseases, it is important to understand the role of Wnt signaling, whether canonical or non-canonical, during cardiogenesis. This is critical especially since studies have demonstrated that differentiation of stem cells into cardiomyocytes imitates the stages of cardiogenesis. Additionally, further examining the crosstalk between the differentiating cardiac cells and components of the extracellular matrix prior to injection could favor the process of cardiac regeneration, and accelerate the application of stem cell therapy for congenital heart diseases.

review, the suppression of the Wnt/β –catenin pathway was found to be the mechanism responsible for the fibro-adipocytic replacement of cardiomyocytes, the characteristic feature observed in ARVC [70]. The authors depicted that suppressed canonical Wnt signaling leads to the differentiation of a group of SHF CPCs in the epicardium to adipocytes due to enhanced expression of adipogenic factors [70]. These adipocytes appear to originate from the SHF progenitor cells, suggesting that ARVC is a disease of cardiac stem cells [69,70].

From a phenotypic perspective, the expression of ARVC mainly involves mutations in desmosomal proteins, which are complex intercellular junctions responsible for cell-cell adhesion and also function as signaling proteins [70]. Desmosomes are made of cadherins, whose extracellular segments bind to each other, while the intracellular segments bind to the anchor protein connected to actin filaments of the cardiomyocyte sarcomere [21]. This dynamic complex is necessary to provide strong mechanical attachments between cardiomyocytes [70] and holds the cells tightly together during contraction [21]. In ARVC, mutations in desmosomal proteins interfere with efficient and proper assembly of desmosomes, allowing the desmosomal protein plakoglobin (PG) to translocate from desmosomes to the nucleus [70]. Ultimately, this interferes with the conventional assembly of the Wnt canonical signaling protein complex, the net effect of which is the removal of the inhibitory effects of the canonical Wnt signaling and increased expression of adipogenesis promoters, thus stimulating the differentiation of a subset of SHF CPCs to adipocytes [69].

Although ARVC is usually diagnosed clinically between 20 and 40 years of age, isolated cases have been observed early in life including reports of right ventricular aneurysm and arrhythmias observed in utero [70]. This suggests that the transcriptional switch from myogenesis to adipogenesis could occur early during cardiogenesis, especially since the disease is now thought to be of cardiac stem cell origin [69]. It has already been established that the stem cell niche microenvironment provides extracellular cues that allows the cells to survive and maintain a balance between quiescence, self-renewal and differentiation [71]. Interestingly, cadherins, along with integrins, are not only key for cell-cell adhesion, but are also involved in cell-ECM linkage and signaling events [65]. Moreover both cell–ECM and cell–cell adhesions share signaling molecules and cytoskeletal linkages, which help regulate cell migration, proliferation, as well as differentiation and morphogenesis [65]. Since the niche is thought to provide protection for stem cells from the accumulation of genetic mutations [71], this suggests that dysregulation of the stem cell niche could play a key pathogenic role in diseases such as ARVC and CHDs.

In the case of ARVC where disease causing mutations in the desmosomal proteins (including cadherins) have been found, this could prompt a direct link to the implications of ECM and stem cell niche disruption and the onset of heart disease in utero. In fact, this observation prompted researchers at the Johns Hopkins University School of Medicine to model ARVC using iPSC-derived cardiomyocytes (iPSC-CMs), in hopes of developing better therapies for this life-threatening disease [72]. Interestingly the authors were able to recapitulate the defects of ARVC by inducing adult-like metabolism in the relatively immature iPSC-CMs. These findings highlight the importance of evaluating the potential of stem cell therapy in ARVC and CHDs in view of molecular genetics of the developing embryonic heart, and the pathways undertaking the development of cardiac myocytes from ESCs, such as the Wnt signaling pathways. Hence, it is becoming increasingly essential to take into consideration the stem cell niche and the mechanisms by which the ECM and its components affect stem cell behavior and function.

9. Conclusions

Considering the importance of Wnt signaling in cardiogenesis, cardiac phenotype specification, as well as CHD, inappropriate Wnt signaling could be the culprit underlying the poor success rate of stem cell therapy in CHD (Fig. 4). Evaluation of current stem cell therapeutic interventions must take into account the relevance of these signaling pathways. Although the current focus of research is to produce more proficient methods for cell administration into neonatal injured hearts, and to improve graft cell survivals and safety, more in depth studying of this critical pathway, and its downstream effectors is of paramount importance. It is also crucial to further examine the crosstalk between the differentiating cardiac cells and components of the ECM, as well as Wnt signaling, which could ultimately have a clear impact on their fate. Together, this could propagate better appreciation for the role of embryonic and microenvironmental cues that favor cardiac regeneration, and effective translation of the outcomes of the preclinical studies into a clinically relevant perspective.

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Authors' contributions

Conceptualization: A.Z.; Writing - original draft: I.A.M., A.Z.; Writing - reviewing and editing: I.A.M., A.Z., N.E.-B.; Supervision: N.E.-B.; Project administration: A.Z., N.E.-B.; Funding acquisition: N.E.-B.

Declaration of competing interest

The authors declare that they have no competing interests.

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