Review

Development of heart muscle-cell diversity: a help or a hindrance for phenotyping embryonic stem cell-derived cardiomyocytes

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Abstract

Despite the advances in cardiovascular treatment, cardiac disease remains a major cause of morbidity in all industrialized countries. The extraordinary potential of (embryonic) stem cells for therapeutic purposes has revolutionized ideas about cardiac repair of diseased cardiac muscle to exciting stages. This, in turn, has challenged research on cardiac differentiation of stem cells. For instance, cultures of mouse embryonic stem cells quite easily differentiate into the cardiogenic lineage, as assessed by their potential to beat spontaneously. However, repair of impaired cardiac muscle by spontaneously beating cardiac muscle cells might impose severe risks upon a human patient. Therefore, it is of crucial importance to understand the mechanisms that underlie the development of the distinct cardiac muscle cell types of the adult mammalian heart. In this review we tried to relate cardiac morphogenesis to the development of unique molecular phenotypes of cardiomyocytes. This relationship will provide a framework to assess the significance of the molecular phenotypes that are observed in embryonic stem cell-derived cardiomyocytes (ESDCs). Although for the phenotyping of ESDCs a comparison should be made with the phenotypes of the developing heart, so far none of the currently available markers allow unequivocal assignment of subtypes.

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1. Introduction

Congenital and acquired heart disease is the main cause of death in industrialized countries. Understanding the molecular pathways of normal heart development is essential for assessing the molecular basis of congenital heart abnormalities and postnatal cardiac death, and for developing appropriate therapeutic interventions. Cell transplantation has gained considerable attention as a potential novel therapy to increase cardiac function in patients suffering from cardiac infarctions [1–4]; however, it is still controversial [5,6] and not without danger. Potential pitfalls of the use of stem cells are the formation of teratomas [7], the introduction of nodal-like (i.e. spontaneously beating) cardiomyocytes that may result in life-threatening arrhythmias [8], and immuno-rejection. Therefore, it is essential to know the mechanisms that underlie differentiation of stem cells towards the cardiac lineage and subsequently towards the desired cardiomyocyte subtypes, such as atrial and ventricular muscle cells or cells of the conduction system. It is also important to find out how potential cell-transplantation strategies can be made more efficient, safer and more tailored.

In this review we discuss cardiac morphogenesis and the development of distinct molecular phenotypes of the myocardial components of the heart, and assess the options and limitations of phenotyping embryonic stem (ES) cell-derived cardiomyocytes (ESDCs). Although for the phenotyping of ESDCs a comparison should be made with the phenotypes of the developing heart compartments, these phenotypes are not very distinctive using the currently known markers. Therefore, unequivocal subtype assignment of ESDCs is not possible.
2. Early differentiation

During a short period of mammalian development, all cells of the embryo proper or inner cell mass have the capacity to differentiate into every cell type of the organism. With gastrulation, this pluripotency is lost and cells become rapidly committed to their final fate. For instance, just after completion of gastrulation, the cranio-lateral part of the visceral mesoderm becomes committed to the cardiogenic lineage upon signals from the surrounding germ layers [9,10]. During this process, the differentiation factors Fgf8, BMPs, Wnt11, and Wnt inhibitors are essential for induction of heart development and are able to induce the cardiogenic program ectopically in the posterior mesoderm [11,12]. Several heart-associated transcription factors, such as Nkx2.5, Hand1, 2, Srf, Tbx5, Gata4, 5, 6 and Mef2c, become expressed in the cardiogenic region, but expression is not restricted to the cardiogenic mesoderm (reviewed in Ref. [12]). In vitro, differentiated mouse ES cells also express many of these factors [13–16] and develop spontaneously contracting cardiomyocytes, indicating that the appropriate developmental signals are present in the cultures.

Meticulous lineage studies identified the cells that contribute to the vertebrate heart [17,18]. However, the fate of the labeled cells was determined at stages, at which heart formation is not yet completed [19], and at which additional myocardium is still being recruited from adjacent mesenchyme at both the arterial and the venous pole of the heart [17,19–23]. Consequently, these adjacent areas added later were not part of the ‘primary’ heart-forming region originally described but were described as originating from the ‘secondary heart field’ or ‘anterior and posterior heart fields’. This description may suggest that different heart fields exist. An alternative option could be to consider these contiguous areas as a single heart-forming region, in which the ventricular region matures first and the other areas are recruited continuously at both poles of the heart. In the primary (linear) heart tube stage, the heart looks like an inverted Y. The symmetrical arms of this Y form the venous pole and the stem represents the arterial pole (see Fig. 3). In mice, the stem comprises the prospective left ventricle; the right ventricle and the outflow tract are recruited later from the so-called anterior heart field [21]. In chickens, however, the stem represents the right ventricle [19], the left ventricle being added from the symmetrical arms of the Y-shaped heart tube. This heterochrony between species in the development of the distinct cardiac compartments may only suggest a difference in timing of similar processes, rather than a principal difference in the origin of the cardiac compartments.

The first possible overt sign of restriction of gastrulating mesodermal cells to the cardiogenic lineage is the expression of the basic helix–loop–helix transcription factor Mesp1 [24]. To define the fate of cells that once expressed Mesp1, a lineage study was performed by Saga and co-workers [24,25], who generated knock-in mice containing Cre recombinase under the control of the Mesp1 promoter-enhancer, crossed them with lacZ reporter mice (in which the LacZ gene is inactive in the absence of Cre-mediated recombination) and analysed the developmental pattern of LacZ-expressing cells in the resulting embryos. From this study the authors concluded that the cardiogenic mesoderm expressing Mesp1 is pluripotent and contains the precursors for the endocardial/endothelial, the epicardial and the myocardial lineages (Fig. 1). The cardiac cushion mesenchyme derived from the endocardium also expressed the transgene, but pericardial cells (parietal pericardium) did not. It is noteworthy that cardiac cushion mesenchyme largely becomes muscular in later stages of development [26], that endothelial cells can be induced into the myocardial lineage [27], and that pro-epicardial cultures can be converted into the myocardial lineage in vitro (unpublished observations from our own laboratory). The transient expression of sarcomeric proteins found in endocardial cells [28–30] is in line with the results of the above-mentioned studies. However, the data obtained from retrovirus-mediated lineage-tagging studies are difficult to reconcile with a common origin [31]. A possible explanation might be that in the cardiogenic mesoderm the myogenic program is initiated first and that very soon thereafter a subpopulation gets committed to the endocardial lineage, a transition not traced by the viral fate map.

3. Primary heart tube

As a result of the process of folding of the flat embryonic disc, the plan of the body takes shape and the cardiogenic mesoderm folds to form the primary heart tube (e.g. mouse embryonic day (E) 7.5) (Fig. 2). The primary heart tube has an outer myocardial layer or primary

![Fig. 1. Origin and differentiation potential of cells from the cardiogenic lineage. See Section 2 for details.](https://academic.oup.com/cardiovases/article-abstract/58/2/303/341140)
Fig. 2. Early development of the mammalian heart tube. Sagittal sections (a, b, c) and corresponding transversal sections (a′ and b′) illustrate subsequent stages of formation of the heart tube. The linear heart tube develops dorsally from the pericardial cavity (a, a′). A portion of the cardiogenic cells becomes epithelially arranged and becomes the endocardium of the future heart tube. The cardiogenic plate that contains the myocardial and the endocardial cells folds and fuses dorsally. Fusion and finally detachment from the dorsal embryonic wall results in formation of the primary heart tube (b, b′). The cardiac jelly is formed during this process and insinuates between the myocardial and the endocardial concentric tubes. Differential growth results in subsequent looping, forming the S-shaped heart (c). Arrowheads indicate expansion (ballooning) of the atrial (blue) and ventricular (red) chambers at the outer curvature. The inner curvatures (*) are opposite of the ballooning chambers and maintain a primary phenotype (purple) (c).

myocardium, and an inner endocardial layer, which are separated by extracellular material deposited by the myocardium. This layer is called the cardiac jelly and is largely composed of glycosaminoglycans [32]. Although in mouse cardiac development the primary heart tube stage takes about half a day at most, conceptually this stage is of paramount interest for the understanding of the building plan of the four-chambered heart and the development of chambers and conduction system in certain locations.

Reminiscent of the heart of the primitive chordate ancestor, the embryonic vertebrate primary heart tube propels the blood by unidirectional peristaltic contraction waves, indicating dominance of pacemaker activity at the inflow part of the heart [33]. A matching sinusoidal electrocardiogram (ECG) was derived from primary heart tubes in chickens [34]. Peristalsis requires slow conduction of the depolarizing impulse and indeed, low conduction velocities were measured in early embryonic vertebrate hearts [35,36] and few gap-junctions were observed [37]. In agreement with these findings, the cardiac gap-junction proteins connexin (Cx) 40 and 43 that are predominant in the formed heart, could hardly, if at all, be detected in the myocardium of the primary heart tube, at both the protein and the mRNA levels [38–41]. In addition, peristalsis does not require well-developed sarcomeric and sarcoplasmic reticular structures, in contrast to the synchronously contracting chambers of the formed heart [42]. Consequently, the cardiomyocytes of the primary heart tube are characterized by low abundance of sarcomeric and sarcoplasmic reticular transcripts [43].

Although all cells of the primary myocardium possess intrinsic automaticity and have poorly developed gap junctions and sarcomers, which make them similar to the nodal cells in the formed heart, they are not all identical. A clear example is the pattern of expression of myosin light chain (Mlc) 2v that is expressed in the anterior part of the heart tube (comprising the atrioventricular canal, the developing ventricular chambers, the inner curvature and the proximal (not distal) outflow tract) and in later stages also in the caval myocardium [44–46]. Thus, Mlc2v is expressed in a part of the tube that gives rise not only to ventricular chamber myocardium, but also to parts of the atrial chambers and to the atrioventricular node. In fact, Mlc2v seems to respond to antero-posterior cues, which
are only a subset of signals required to achieve ventricular chamber formation at the ventral side of the primary heart tube [46,47]. Despite this, Mlc2v expression is widely used as a paradigm for the specification of the ventricular chambers and of chamber myocytes derived from differentiating ES cells in vitro [48–53].

Many genes whose expression in the adult heart is confined to either the atrial compartment or the ventricular compartment are expressed in gradients along the antero-posterior axis of the heart tube. Generally, ‘atrial’ gene expression preponderates in the upstream (posterior) regions of the tube, while ‘ventricular’ gene expression preponderates in the downstream (anterior) part of the tube. Thus α- and β-myosin heavy chain (Mhc), Mlc1a, 1v and 2a are initially expressed in the entire heart-tube in gradients, and become first restricted to their compartments in the later, foetal stages [44]. Consequently, the expression of none of these genes on its own can be used to ascribe an atrial or ventricular identity to ES cell-derived cardiomyocytes.

4. Cardiac chambers

The evolutionary development of the chamber heart provided the vertebrates with a more powerful heart, in which the atria became the drainage pool of the body allowing efficient filling of the ventricles, and in which the ventricles became the power pump allowing generation of adequate blood pressure. In all vertebrates, the atria develop at the dorsal upstream part of the primary heart tube and the ventricles at the ventral downstream side of the tube (Fig. 3). In other words, the chambers develop at the outer curvatures of the S-shaped heart tube, rather than as an integral part or a segment of the primary heart tube (Fig. 2). Consequently, the myocardium of the original primary heart tube remains present as a contiguous structure from the venous pole to the arterial pole. Conventionally, an inflow part (sinus venosus, inflow tract), an atrioventricular canal, and an outflow tract are distinguished. This subdivision is clear at the outer curvatures where the chambers balloon out, but it is impossible to observe at the inner curvatures [54].

Morphologically and functionally, in vertebrates the chamber myocardium of the developing atria and ventricles can be distinguished from the primary myocardium of the linear heart tube [46,55]. The chamber myocardium becomes trabeculated, whereas the primary myocardium is smooth and covered with so-called cardiac cushions. The functional difference between a primary heart tube and an early looping chicken heart became apparent by the fact that an adult type of ECG could be derived from early looping chicken hearts within 1 day after the formation of the linear heart tube [34,56]. The presence of an adult type of ECG indicates that fast-conducting atrial and ventricular chambers have developed.

Fig. 3. Cardiac chamber formation in mice. The upper panels show a series of cartoons of ventral (E7.5–8, E8.5, E11.5) and left lateral (E9.25) views of the embryonic mouse heart tube and of the four-chambered heart. In the lower panels the spatial and developmental expression profiles of Anf, as visualised by whole-mount in situ hybridisations, show that Anf expression marks chamber formation. V, embryonic ventricle; oft, outflow tract; avc, atrioventricular canal; ift, inflow tract; LA+RA, left+right atrium; LV+RV, left+right ventricle; ap, arterial pole; vp, venous pole.
The clearest markers that in mammals identify the developing chamber myocardium are the atrial natriuretic factor (Anf) and Cx40 genes, which are not expressed in the myocardium of the primary heart tube [39,46,57]. During further development, the trabecular myocardium of the ventricle proliferates rapidly on the epicardial side, by which the compact ventricular myocardium is formed. Anf and Cx40 are not expressed in the compact ventricular layer and become confined to the interiorly localized trabecular component that will later form the bundle branches and peripheral ventricular conduction system [58]. Both genes are expressed in the embryonic atria that will later form the atrial appendages of the formed heart. During further development, the smooth-walled dorsal atrial wall (comprising the pulmonary and caval myocardium) as well as the atrial septa are incorporated into the atria. These components do not express Anf, but do express Cx40. Cx43 displays a pattern of expression that is similar to that of Anf and Cx40, but atrial and ventricular expression is initiated later on during development [39,46].

A gene that is clearly upregulated in the cardiac chambers is sarco-endoplasmic reticulum Ca^{2+} ATPase (Serc2a2) [59], but because it is also expressed in the primary myocardium it is less suited as a marker for the developing chambers. The functional significance of the chamber program of gene expression is that it allows fast, synchronous contractions.

The regionalized formation of the atrial and ventricular chambers requires antero-posteriorly (AP) and dorso-ventrally (DV) distributed direction signals. Retinoic acid seems to be an important cardiac AP-patterning molecule [60,61]. For instance, in quail, deficiency of retinoic acid resulted in poor development of posterior cardiac structures [62,63] and in quail, excess of retinoic acid resulted in 'atrialization' of the heart tube at the expense of the anterior ventricular part [64–66]. In other studies, the transcription factors Tbx5 and Gata4 were upregulated, providing a potential functional link of these factors with AP-patterning. The expression of Nkx2.5 did not change with excess of retinoic acid, which suggested a limited role for this transcription factor in AP-patterning. Concordantly, Nkx2.5 was found to be homogeneously expressed along the heart tube [67,68]. Nkx2.5 must, however, be important for the differentiation of chamber myocardium, because in Nkx2.5-deficient mice that displayed normal cardiogenesis, the formation of chambers failed [69,70]. To study the effects of retinoic acid in the ES cell system the Mic2v promoter region was used [52]. Differentiating ES cell cultures incubated with retinoic acid were reported to display increased promoter activity of the 2.1 kb Mic2v–LacZ transgene [52]. This contradicts the situation in vivo and indicates the difficulties of using the ES cell culture system as a model for cardiogenesis. In vivo, pattern formation along the DV axis has been shown to occur, but the underlying mechanism is not yet clear, nor is the integration of AP and DV signals in controlling the onset of chamber formation. For instance, at the ventral side of the linear heart tube Hand1 is expressed selectively [46,71,72]. Chimaeric mice generated from Hand1 knock-out ES cells and wild-type (ROSA26–lacZ) ES cells showed that, although the Hand1 knock-out cells contributed to the heart, they were never found at the outer curvature in ED 9.5 embryos [73]. This suggests the involvement of Hand1 in DV patterning.

5. Nodal tissues and ventricular conduction system

One of the most fascinating features of the developing mammalian heart is the almost inconspicuous conversion of the peristaltic primary heart tube without valves into the synchronously contracting chamber pump with valves. Poor coupling of the myocardial cells of the primary heart tube results in slow conduction of the depolarizing impulse, which in turn causes peristaltic contraction waves. The long duration of this type of contraction prevents backflow of blood and valves are dispensable. A chamber heart needs one-way valves at both ends of the chambers to prevent refilling from the downstream compartment during relaxation, and to prevent backflow to the preceding compartment during contraction. The gradual formation of chambers from the primary heart tube guarantees that the forming chambers are flanked by primary myocardium that functions as a sphincter and substitutes valve function. It is remarkable that the phenotype which permits the slowly travelling waves of peristaltic constrictions along the primary heart tube is a prerequisite for the pacemaker (nodal) function as well. Intercellular coupling of nodal cells needs to be low, permitting loading of the cells to a level sufficiently high to drive the huge myocardial mass of the chambers [74]. For this reason, it is not coincidental that the cardiac nodes develop in areas where the valves develop as well; this holds true for the sinu-atrial and atrioventricular regions. Recent studies from our laboratory have demonstrated that Tbx2 is involved in the repression of Anf promoter activity in the atrioventricular canal region that retains its primary myocardial phenotype [75], which indicates a role for Tbx2 as an inhibitor of chamber specification.

6. The main phenotypes of the heart cells and consequences for ESDC phenotyping

Based on both morphological [42] and electrophysiological [76] criteria, four main phenotypes of cardiomyocytes that arise during development of the mammalian heart can be distinguished (Table 1). All cardiomyocytes have sarcomeres and a sarcoplasmic reticulum (SR), are coupled by gap junctions, and display automaticity. Differences in the degrees to which these properties are present determine the cardiomyocyte sub-
Table 1
Morphological and functional features of the main subtypes of cardiomyocytes

<table>
<thead>
<tr>
<th>Feature</th>
<th>Cardiomyocyte subtype</th>
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<tbody>
<tr>
<td></td>
<td>Primary</td>
</tr>
<tr>
<td>Automaticity</td>
<td>High</td>
</tr>
<tr>
<td>Conduction velocity</td>
<td>Low</td>
</tr>
<tr>
<td>Contractility</td>
<td>Low</td>
</tr>
<tr>
<td>SR-activity</td>
<td>Low</td>
</tr>
</tbody>
</table>

types. Cells of the primary heart tube are characterized by high automaticity, low conduction velocity, low contractility, and low SR activity. This phenotype largely persists in nodal cells. In contrast, atrial and ventricular working myocardial cells display virtually no automaticity, are well coupled intercellularly, have well developed sarcomeres, and have a high SR activity. Cells from the atrioventricular bundle, bundle branches and peripheral ventricular conduction system have poorly developed sarcomeres, low SR activity, but are well coupled and display high automaticity.

Apart from the above-mentioned criteria, the different cardiac compartments have unique molecular phenotypes (Table 2) [44,45,58]. The morphological, functional and molecular characteristics that are relatively well-distinguishing features in adult cardiomyocytes gradually develop during cardiogenesis; therefore none of them can be used individually for subtyping of ESDCs.

ESDC phenotyping has been based mainly on expression analysis in homogenates of differentiated mouse ES cells and on measurements of electrical properties predominantly in isolated ESDCs. By comparing the outcomes of these studies with the phenotypes of adult heart cells, the suggestion was raised that ESDCs develop characteristics of working myocardium of the chambers (see below). We believe that for ESDC phenotyping comparison with the developing heart is crucial, but that when this is done, alternative interpretations remain open. In the following, we discuss the conclusions from previous studies, taking into account the phenotypes of the developing heart.

6.1. Molecular characterization

For α-Mhc, β-Mhc and cardiac Troponin I and slow skeletal Troponin I developmental transitions were observed in differentiated ES cell cultures [77,78]. These transitions were interpreted as reminiscent of the shifts that occur in vivo around birth or at the end of gestation. However, because all these isofoms were heterogeneously expressed along the entire heart tube, shifts in expression of these genes in ESDCs do not necessarily imply differentiation into more mature cells [44,46]. The β- to α-Mhc shift in rodents is a birth-associated phenomenon related to the change in thyroid hormone status [79–81] that is unlikely to occur during ES cell differentiation. Moreover, expression of Mlc2v and Anf is often used to demarcate ventricular-like [49–53] and atrial-like [82,83] cells in ES cell cultures, respectively. As mentioned in Section 3, Mlc2v is already expressed very early during heart development before chamber formation is morphologically initiated, and during development is not exclusively restricted to parts that develop into the ventricular chambers. Therefore, expression of Mlc2v in ESDCs is not necessarily linked to chamber specification. To use Anf expression in ES cell cultures is interesting, because Anf expression identifies the differentiation of a new cardiac phenotype. In the embryonic mouse heart, Anf expression exclusively identifies the forming atrial and ventricular chambers. It is never expressed in nodal tissues, and it is only expressed in the atrial appendages but not in the rest of the atria in the adult heart [45,46,84]. Therefore, in ESDCs, Anf expression does not exclusively identify atrial cardiomyocytes. For this reason we believe that Anf can be used as a marker for differentiation of the working myocardial cells in ES cell cultures. Several groups have reported Anf expression in ESDCs both at the mRNA level and at the protein level [82,83]; however, mRNA expression is low and comparable to the level found early on during heart development [43].

Table 2
Expression of some distinguishing genes in different compartments of the developing heart

<table>
<thead>
<tr>
<th>Cardiac component</th>
<th>Cx40/43</th>
<th>Anf</th>
<th>Mlc2v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflow tract * (SAN)</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Embryonic atrium</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Atrioventricular canal * (AVN)</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Embryonic ventricle</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Outflow tract</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrium</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ventricle</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

*Sinu-atrial and atrioventricular nodes (SAN and AVN) develop in these embryonic regions.

* In the foetal period Mlc2v becomes expressed in the inflow tract [44].

* Mlc2v is only expressed in the proximal part of the outflow tract. The adult atrium originates from the inflow tract, the embryonic atrium, and the atrioventricular canal. The adult ventricle originates from the embryonic ventricle and the outflow tract.
6.2. Electrical properties

Electrophysiological characterization of ESDCs for phenotyping is mainly based on the shape of the action potentials and the effect of selective drugs on this shape [51,52,82,85–87]. Although at first glance phenotyping seemed straightforward, unambiguous classification of these cells was difficult because subtle differences demarcated subtypes and extensive variation in typical action potentials was observed [52,85]. Proper determination of the progress of differentiation based on electrophysiological features requires a reference series of these parameters derived from the distinct compartments of the embryonic heart at different developmental stages. At present, only a few studies have been published on electrophysiological measurements in mice [51,88]. Shapes of action potentials were not measured and the compartmental origin of the cardiomyocytes was not described. Therefore, the 'normal' variation of cardiomyocyte diversity in the mammalian linear heart tube is as yet unknown.

In conclusion, gene-expression studies on differentiating ES cells are still in an embryonic stage. However, so far, the reported data are in agreement with a limited progress of differentiation.

7. Perspectives

The interplay of several molecular pathways in a precisely orchestrated spatio-temporal context results in the formation of the four-chambered mammalian heart. At present only some key molecular mechanisms are known [12,47]. It is fascinating and surprising that cardiogenesis can be initiated in a culture model of mouse ES cells with relative ease, but whether or not the differentiation of all the myocardial subtypes can be achieved in vitro remains to be established. The ES cell culture model is potentially an important tool to test in vitro the role of regulatory gene products in cardiac differentiation. As reviewed by Boheler et al. [89], these experiments should be interpreted with care, because clonal variance, promoter dependence and possible silencing of ectopically expressed transgenes in individual ES cells, could adversely affect interpretation of results.

Much of what we know about cardiomyocyte differentiation in the ES cell system has been learned from mouse ES cells. Human ES cells are currently extensively studied to find out whether they are comparable to those of the mouse. In vitro, cardiogenesis of human and mouse ES cells is broadly comparable, although culturing and differentiation of the human ES cell lines appears to be more difficult than their mouse counterparts [90]. Therefore, it is as yet uncertain whether human ES cells will be as versatile as mouse ES cells regarding self-renewal, genetic manipulation and developmental potential.

Since advanced differentiation of ES cells has not been proven yet, studies to reveal pathways underlying differentiation into chamber myocardial cells are important to eventually be able to develop donor cells for transplantation. In this context the role of T-box transcription factor (Tbx) family members is of great interest. They comprise a large family of DNA-binding proteins and are important components of several developmental pathways that specify structures and organs, including the heart. For instance, heterozygous mutations of Tbx factors cause congenital malformations in humans [91–95]. Tbx5 haploinsufficiency resulted in the Holt–Oram syndrome, in which both human patients and mutant mice showed upper limb and heart malformations, such as septal defects and conduction disturbances [96]. The analysis of Tbx5 and Tbx2 is interesting, because Tbx5 works as a positive regulator of early cardiogenesis and chamber specification [96–98], whereas Tbx2 represses components of the chamber-specific program of gene expression [75].

Although increasing amounts of data on molecular and electrophysiological profiles have become available, divergent opinions on the progress of maturation of ESDCs exist. This may find its origin in the comparison of ESDCs with the adult situation only. Taking the embryonic situation into account as well may be a fruitful way to a better understanding of the differentiation process in vitro, and may eventually even result in clinical applications.

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