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Review

Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis

J. Hescheler [a,*], B.K. Fleischmann [a], S. Lentini [b], V.A. Maltsev [d], J. Rohwedel [c], A.M. Wobus [c], K. Addicks [b]

[a] *Institut für Neurophysiologie, Universität zu Köln, Robert-Koch Strasse 39, D-50931 Köln, Germany*
[b] *Institut für Anatomie I, Universität zu Köln, Josef-Stelzmann Strasse 9, D-50931 Köln, Germany*
[c] *Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstrasse 3, D-06466 Gatersleben, Germany*
[d] *Henry Ford Heart and Vascular Institute, 2799 West Grand Blvd., Detroit MI 48202, USA*

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1. Introduction

In order to study cardiac myocyte development different approaches were established during the last decades. The main purpose of these studies was the differentiation of cardiac precursor cells into specialized, differentiated cell types, as well as the development of functional properties such as Ca^{2+} handling, rhythm generation and excitation-contraction coupling of cardiomyocytes during development. Although considerable data exist about skeletal myogenesis [1–3], limited knowledge is available with regard to the origin of the commitment and differentiation of cardiac cells. A comprehensive, morphological study on the cytodifferentiation from mesenchymal cells into cardiac myocytes is described in the embryonic murine heart [4]: According to the authors, different stages of myofibrillogenesis are present during embryological myocardial development. Cells with no or only little myofibrillar arrangement develop to myocardial cells with orientated myofibrils [5,6]. A number of morphological studies have investigated heart development on embryonic, neonatal and adult isolated cardiomyocytes also from different species [7–16].

Although the ultrastructure during cardiac development has been thoroughly investigated [17], still relatively little is known on the development of excitability of the mammalian heart, most importantly: (1): The relation between expression of cardio-specific genes (see review [23]), the formation of cardiac phenotypes and the functional expression of different types of ion channels; (2): The regulation and genetic control of expression of ion channels (e.g. by growth factors, hormones, extracellular matrix); (3): The development of the regulation of ion channels and morphological correlates. The progress in this field is hampered by the inability to study cardiomyocytes from early, embryonal hearts because of their very small size and because of the lack of cardiac cell lines that mimic various stages of cardiac development. The development of ion currents has been studied in cardiomyocytes prepared from mammalian embryos not earlier than shortly before birth [18–21], but see Ref. [22]. Cells of this developmental stage already exhibit action potentials of highly differentiated cardiomyocytes (e.g. of ventricular-like type, [19]) and thus can serve as a model for studying only late stages of prenatal heart development.

The focus of this review is on the development-dependent changes of morphology and electrical activity during cardiomyogenesis. Since the amount and orientation of myofibrils allows the classification into the different stages of myocyte development, the morphological part deals in detail with the myofibrillogenesis. The combination of the analysis of the electrical membrane properties and morphological criteria allows the correlation of morphological and functional phenotypes. These aspects of cardiac myogenesis are studied in a model based on the differentiation of pluripotent murine embryonic stem (ES) cells into cardiomyocytes in vitro.

2. Cell lines to study cardiomyocytes

Many studies deal with primary myocardial cultures of mammals demonstrating ultrastructural and functional fea-

* Corresponding author. Tel.: (+49-221) 4786960; Fax: (+49-221) 4786965.

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tures of the terminally differentiated heart (for review see [24]). The cell-isolation techniques used to obtain these primary cultures are, however, not suitable for prenatal, early cardiac developmental stages due to the small quantity of myocardial material and the difficulty to maintain the developmental stage within the cell culture procedure. Therefore a first attempt to generate permanent cell lines of early cardiac differentiation was undertaken in the late 70's:

1. A permanent cell line, H9c2 derived from embryonic rat heart [25], exhibited dihydropyridine sensitive L-type Ca^{2+} channels activated by isoproterenol, presumably through the G_s α-subunit and the cAMP-dependent pathway [26]. In addition, H9c2 cells express two distinct K^+ channels and a nonspecific cation channel [27]. Furthermore, both single channel studies and RT-PCR analysis with α_1 isoform-specific primers revealed an expression of skeletal L-type Ca^{2+} channels in addition to the cardiac type of the channel [28].
2. Immortal cardiac cell lines have been established from embryonal avian heart [29] and adult rat myocardium [RCVC, [30]]. Under appropriate cultivation conditions these cell lines expressed some muscle-specific markers (e.g. α-sarcomeric-actinin, α-actin, desmin). Moreover, the immortal cell line established by Caviedes and coworkers [30] exhibited inward currents that resemble T- and L-type Ca^{2+} currents. In spite of the recent progress, the usage of these permanent cell lines is limited because they retain only a few markers of differentiated heart cells and lack many other important characteristics such as contraction and generation of action potentials.
3. A new approach based on transgenic mice with myocardial tumors has been developed to establish cardiomyocyte cell lines (see review [31]). The cultured cardiomyocytes derived from tumors (e.g. AT-1) can be maintained in culture, but have to be passaged into mice for regeneration. These cells express some skeletal muscle- and cardiac-specific markers and retain a highly organized ultrastructure and exhibit contractile activity as well as action potentials similar to those generated by normal cultured atrial cells.
4. Proliferating cardiac cells obtained by transfection with SV-40 large T antigen [32] also retain certain differentiated properties including myosin light chain expression and assembly into organized myofibrils, spontaneous contractile activity and chronotropic responses to adrenergic agonists.

The disadvantage is, however, that the cardiomyocytes derived from myocardial tumors or by virus transfection with SV-40 large T antigen can be passaged only for a limited time. The lack of other surrounding cells forming the natural microenvironment for cardiomyocytes impairs the use of these cell lines to model the in vivo cardiomyogenesis. It is also known from the literature that cellular contacts [34,35,11] and the extracellular matrix [33,10,8] influence myofibril assembly and myocyte cytoarchitecture. The loss of real three-dimensional geometry and cellular matrix relation in culture may therefore induce significant changes of the cardiomyocyte phenotype.

3. Factors influencing cardiac myogenesis

In contrast to the relatively accurately described functions of growth factors in skeletal myogenesis [36] and of extracellular matrix in the cardiovascular system [37], their role in cardiomyogenesis is not well characterized (for review see [38]). The role of growth factors in cardiac development has been mostly investigated in embryological hearts: (i) tumor growth factor β1 (TGFβ1) stimulates cardiac mesoderm formation [39], (ii) β fibroblast growth factor (βFGF) is involved in the autoregulatory processes of cardiomyocyte proliferation and differentiation [40] and in processes of down-regulation of initial stages of the vertebrate cardiac development in vivo [39]. However, with these models a direct experimental approach to study the underlying signal transduction cascades is hampered because of the complexity of the whole embryo. Experi-

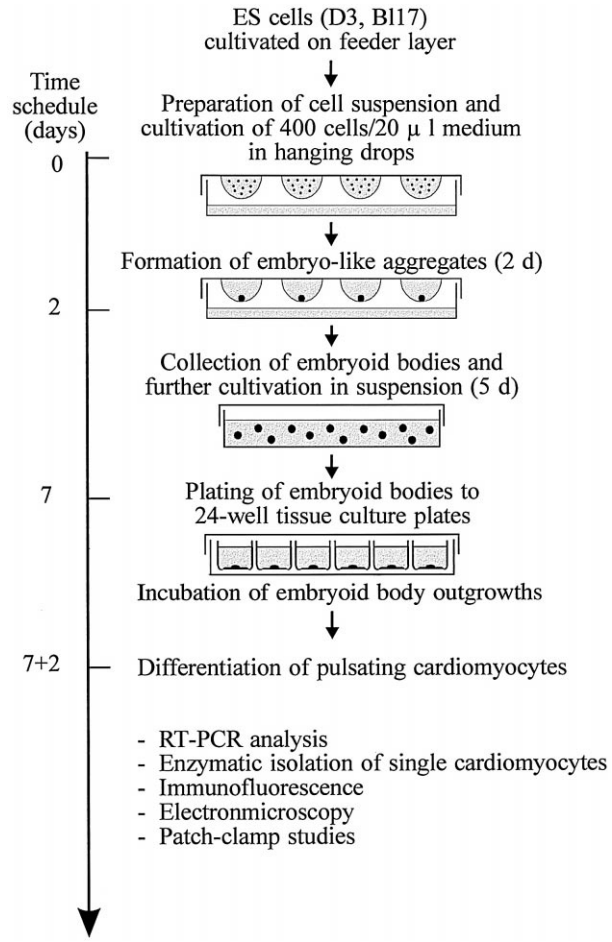

Fig. 1. Cultivation protocol for the generation of ES-cell derived cardiomyocytes.

ments with insulin growth factors (IGFs), which are found to bind to receptors in plasma membranes of embryonic chicken heart tissue [41], as well as studies demonstrating platelet derived growth factor (PDGF)-receptors during somatogenesis localized in the murine heart implicate the importance of tyrosine-kinase-receptors in heart development.

4. The embryonic stem (ES) cell model

A new approach to study cardiomyogenesis is provided by the use of pluripotent murine embryonic stem cells (ES cells, ESC) differentiated in vitro. The ES cells originally have been derived from undifferentiated cells of the inner cell mass of murine embryos at the blastocyst stage or from eight-cell embryos [42–44]. They are kept in permanent culture if grown on feeder layer cells (embryonal murine fibroblasts) or leukemia inhibitory factor (LIF) [45]. ES cells are capable to take part in the embryonic development in vivo after retransfer into blastocysts (e.g. [46]). In vitro, ES cells have been shown to differentiate spontaneously into derivatives of all three primary germ layers, endoderm, ectoderm and mesoderm [47–50].

The principle of differentiation is schematically illustrated in Fig. 1:

1. the cultivation of a definite number of cells (e.g. 400 cells) in 'hanging drops' (20 μl) as embryoid bodies (EBs) for 2 days,
2. cultivation in bacteriological dishes for 5 days,
3. plating of '7d'-EBs for further differentiation.

Similar to in vivo development EBs consist of cellular phenotypes derived from the three germ layers forming a complex multicellular arrangement. Among others, skeletal [51] and smooth muscle cells, neuronal cells [52,53] forming networks with synaptic connections, glial cells (micro- and macroglial) [54], blood vessels (containing endothelial and smooth muscle cells) [55,56], hematopoietic cells, glandular cells (Addicks, unpublished data) as well as epithelial cells [57] form organ-like structures. Beside these different mesenchymal cells, components of extracellular matrix like different types of fibre collagen, laminin, nido-

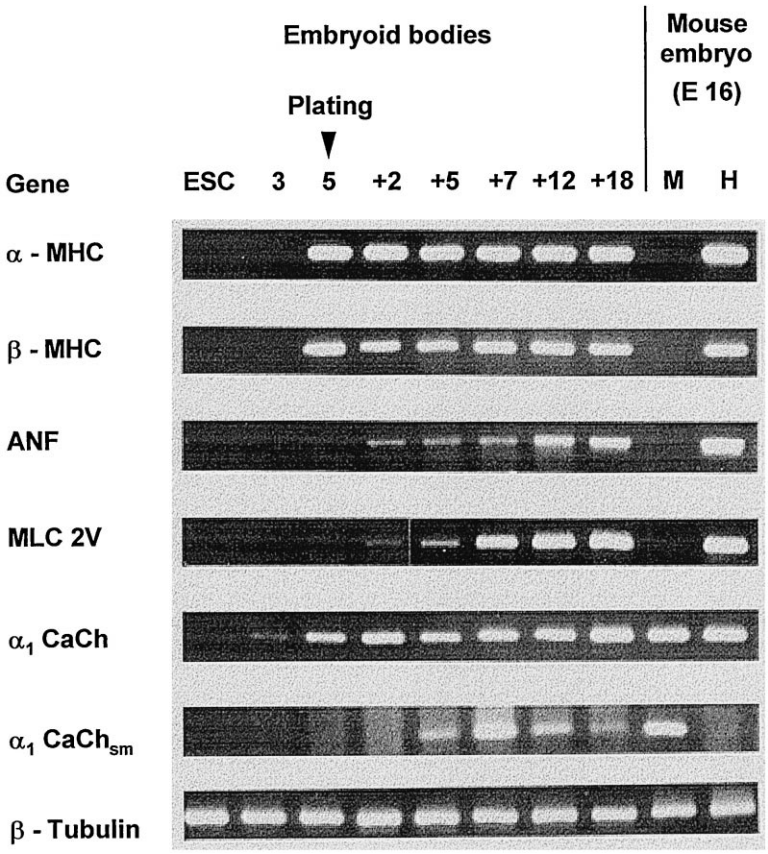

Fig. 2. Expression of cardiac- and skeletal muscle-specific genes during in vitro differentiation of ES cell (line D3)-derived embryoid bodies plated at day 5 as analyzed by RT-PCR. A developmentally controlled expression pattern of the cardiac-specific genes encoding α- and β-cardiac myosin heavy chain, ANF, and myosin light chain isoform 2V was found with the latter two being upregulated during cardiac-specific specification stage. The first cardiac-specific transcripts of the gene coding for the α_1-subunit of the L-type Ca^{2+} channel (α_{1CaCh}) were already detected at day 3, two days before the first beating cells appeared. Skeletal muscle-specific genes encoding the skeletal muscle-specific α_1-subunit of the L-type Ca^{2+} channel ($\alpha_1 CaCh_{sm}$) were found to be expressed at a stage when the first myocytes appeared in the embryoid body outgrowths. Primers specific for the house keeping gene β-tubulin were used as an internal standard, and skeletal muscle (M) and heart (H) from a day 16 p.c. mouse embryo as positive controls.

gen and fibronectin form the connective tissue in the EB [58,59] like in vivo.

Within this multicellular arrangement in EB outgrowths (from 7 + 1d to 7 + 24d) cardiomyocytes appeared as spontaneously contracting cell clusters. They increased in size during further differentiation. The cluster can be investigated in total, or dispersed into single cells by aid of widely used isolation techniques. Because of the small number of cell layers forming the total cellular mass of the outgrowth, the EB is transparent for light and therefore can be permanently observed and controlled by aid of vital microscopy.

A developmentally controlled expression pattern of the cardiac-specific genes encoding α- and β-cardiac myosin heavy chain (α-, β-MHC), atrial natriuretic factor (ANF) and myosin light chain isoform 2V (MLC-2V) can be detected by RT-PCR of the total mRNA of EB's (see Fig. 2): While α- and β-MHC expression remains constant the latter two are upregulated during the cardiac specification stage. The first cardiac-specific transcripts of the gene coding for the α_1 subunit of the L-type Ca^{2+} channel (α_1 CaCh) are already detectable at day 3, two days before the first beating cells appear [58]. Skeletal muscle-specific genes encoding the myogenic regulatory factors myogenin

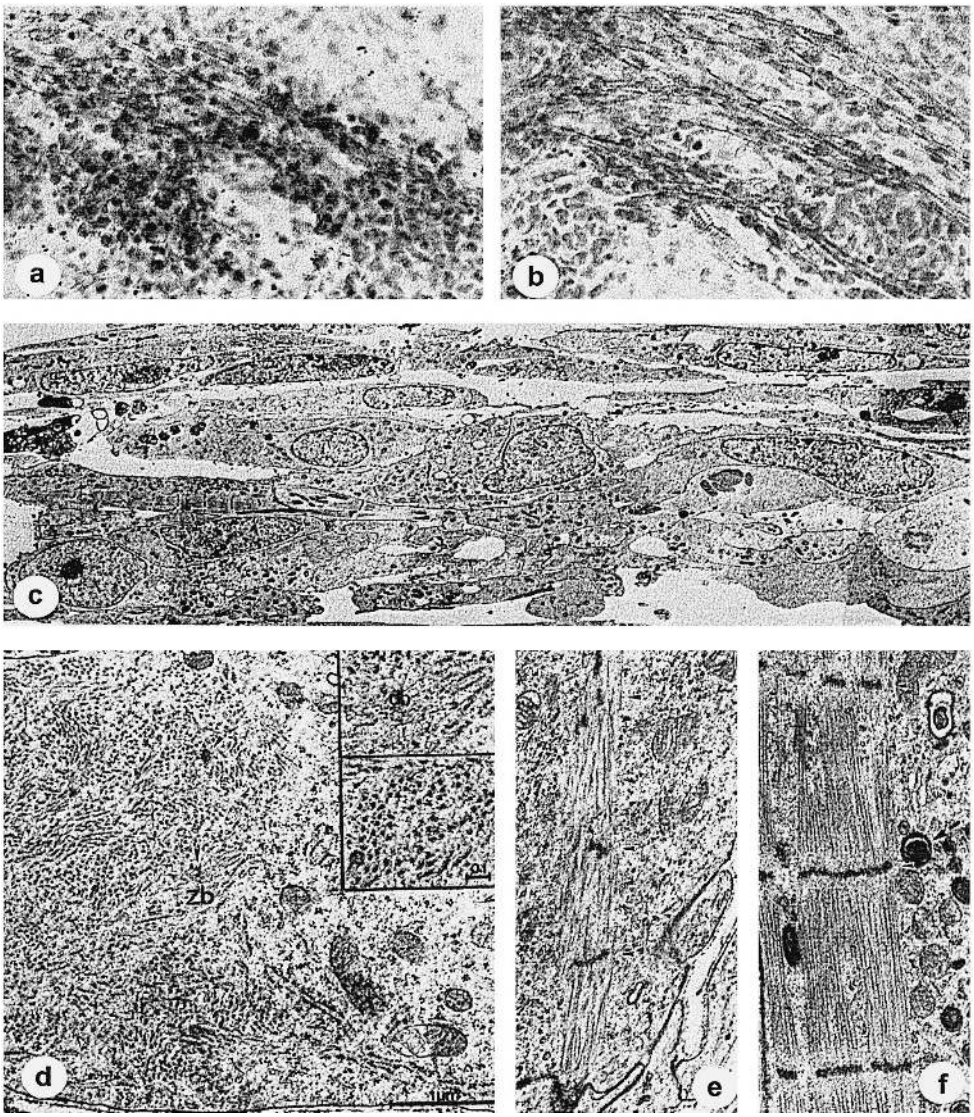

Fig. 3. Cardiomyocytes in a and b are identified by α-sarcomeric-actinin labelling. (a) Early stage EB (7 + 2d): Small rounded early myocytes form round accumulations. (b) Late stage EB (7 + 12d): Elongated cardiomyocytes with well developed myofibrils are situated in strands. (c) The cardiomyocyte aggregates within the EB are located between a covering epithelial layer and a basal layer of mesenchymal cells. (d) Early stage EB (7 + 2d): Actin and myosin filaments form alternating bundles and are in a irregularly and disorientated manner distributed in the cytoplasm. Z-bodies (zb) are rare. The cross-section demonstrates a hexagonal array of actin and myosin filaments. (e) Early stage EB (7 + 4d): Z-bodies are aligned periodically and are associated with actin filaments. The Z-bodies are interrupted by bundles of myosin filaments that are located between two dense bodies. Further development of the myofibrillar arrangement shows lateral alignment of several primitive myofibrils and fusion of Z-bodies to Z-line precursors which remain irregularly shaped. There is still no clear distinction of I- and A-bands. (f) Late stage EB (7 + 12d): The diameter of the Z-line corresponds to the diameter of the myofibrillar arrangement. Z-line, I- and A-band are distinct, but in comparison to adult cardiomyocytes the M-band is still missing.

and MyoD as well as the cell adhesion molecule M-cadherin and the skeletal muscle-specific α_1-subunit of the L-type Ca^{2+} channel (α_1CaChsm) are found to be expressed in a stage when the first myocytes appeared in the EB outgrowths [51].

5. Morphology of ES cell derived cardiomyocytes

The formation of developing cardiomyocytes in EBs represents an in vitro tool demonstrating a progredient differentiation of cardiomyocytes from pluripotent ES cells. In early stages of EB development (7 + 2d) the spontaneously contracting areas consist of small rounded early-stage myocytes, which are situated in round accumulations (Fig. 3a). With maturation of such cardiomyocyte formations, the overall appearance changes to strands of elongated cardiomyocytes with well developed myofibrils (Fig. 3b). The arrangements of cardiomyocytes within the EB are located between a covering epithelial layer and a basal layer of mesenchymal cells. The cardiomyocytes are surrounded by a discontinuous basal lamina and the myocytes are linked by cellular contacts (Fig. 3c). Hence, cardiomyocytes of the EB are always in a structural and functional relationship to other cells and extracellular matrix proteins.

Although in the mature heart atrial granules (atrial natriuretic factor, ANF) containing cells are preferentially found in the atria [60], most of the cardiomyocytes developed within EBs contain these atrial granules [61], suggesting an important role of ANF in early cardiac development. Like in the developing heart, T-tubuli are not promi-

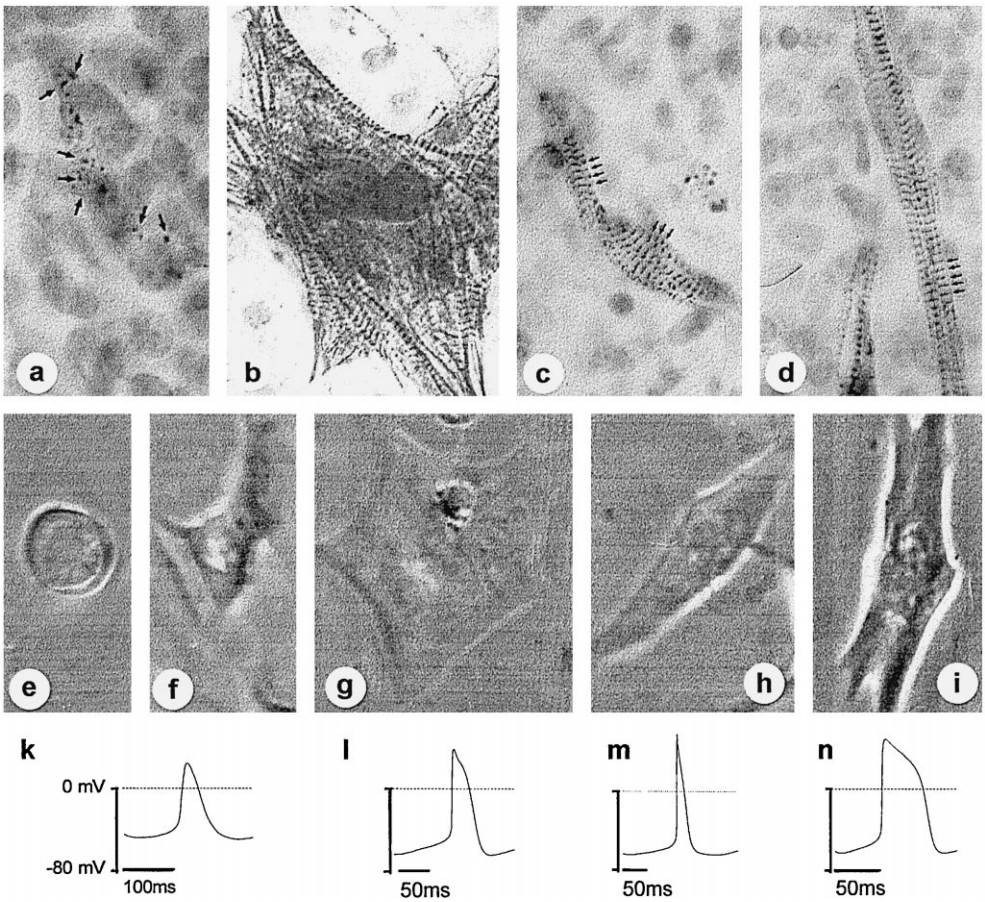

Fig. 4. At the single cell level EB derived cardiomyocytes can be classified based on overall shape and myofibrillar organization into early, pacemaker-like (see a, e, f, and j), Purkinje-like (b, g, and k), atrial-like (c, h, and l) and ventricular-like (see d, i, and m) cells. (a) Early cells (Fig. 4e) with no or rarely developed sarcomeres are relatively small (35 μm diameter) and display a round to branched shape. (b) Purkinje-like cells (see Fig. 4b, g) are large with one or two round nuclei and show a highly branched shape. The peripherally placed myofibrils are aligned in the direction of the tension forces. In contrast to freshly dissociated ventricular myocytes, the typical parallel orientation of the myofibrils is not present. (c) The atrial-/ventricular-like cells are elongated with an oval nucleus and well organized tension-orientated myofibrils, spanning the long axis of the cells. (e–i) The characteristic morphological pattern of EB derived cardiomyocytes can also be distinguished by vital microscopy into early pacemaker-like cells (e, f), pacemaker-like cells, Purkinje-like cells (g), atrial- (h) and ventricular-like (i) cells. (k–n) Representative action potential recordings from spontaneously beating, single cardiomyocytes in the current-clamp mode: (k) Relatively depolarized cardiomyocyte with short lasting AP of small amplitude characteristic for an early, pacemaker-like cell. (l) Cell with a relatively stable resting potential, notch and plateau phase characteristic for a Purkinje-like cell. (m) Cell with a relatively stable resting potential and short lasting AP typical for an atrial-like cell. (n) Cell with relatively stable, negative resting potential and long plateau phase typical for a ventricular-like cell.

nent in all stages of cardiomyogenesis within EBs. It has been shown that the T-tubular system only develops postnatally [62,63]. Adjacent cardiomyocytes show different degrees of myofibrillar organization. Also, within the same cell, different stages of myofibrillar assembly, leading to the definite sarcomeric architecture, can coexist.

Corresponding to the cell shape, the α-actinin positive sarcomeric structures differentiate from spots in roundish cells (early and pacemaker-like cells) to complete sarcomeric structures and oriented myofibrils in elongated cells (differentiated atrial- and ventricular-like cells). In respect to the arrangement of filamentous elements and the appearance of an amorphous material (comparable to the Z-line material because of the electron density), the following patterns of myofibrillar assembly in cardiomyocytes can be distinguished (Fig. 3a–f) [61]:

1. Spots of Z-line material (Z-bodies) are dispersed in the cytoplasm.
2. Actin- and myosin filaments form alternating bundles which are irregularly and in a disoriented manner distributed in the cytoplasm. The cross-section demonstrates a hexagonal array of actin- and myosin filaments. The Z-bodies are seldom.
3. The Z-bodies are aligned periodically and associated with actin filaments. The Z-bodies are interrupted by bundles of myosin filaments which are located between two dense bodies; these have a greater diameter than the Z-bodies.
4. Further development of the myofibrillar arrangement shows lateral alignment of several primitive myofibrils and fusion of Z-bodies to Z-line-precursors which remain irregularly shaped. There is still no clear distinction of I- and A-bands.
5. The diameter of the Z-line corresponds to the diameter of the myofibrillar arrangement, the spacing between two Z-lines varies between 1.7 and 2.5 μm. The Z-line, the I- and the A-band are distinct, but in comparison to adult cardiomyocytes the M-band is still missing. The M-band formation is considered the endpoint of myofibrillar maturation [64,65] and is presumably related to the mechanical requirements in the heart (pressure load). Similar mechanical functions are not required in the EBs and may explain the lack of the typical M-band formation.

6. Diversity of cardiac cell types in the EB

As shown also by the electrophysiological measurements (see below) the heterogeneous population of cardiomyocytes undergoes a shift from early stage cardiomyocytes with pacemaker activity to terminally differentiated atrial-/ventricular-like cells. This is from the morphological point of view due to the shift from small rounded cells with low myofibrillar content to elongated cardiomyocytes with high content of organized myofibrils.

On the single cell level the cardiomyocytes derived from EBs can be classified as early pacemaker-like, Purkinje-like and atrial-/ventricular-like cells in regard to their overall shape and myofibrillar organization stage [61]. The cells can be clearly classified in these cell types, even though morphological intermittent stages are observed (Fig. 4):

- Early cells (Fig. 4e) with no or rarely developed sarcomeres are predominantly small and round (35 μm diameter).
- Pacemaker like-cells (Fig. 4a,f) vary in size from the smallest to the largest cells in the cardiomyocyte arrangements and also in shape from round to branched cells. The typical pacemaker-like cell shows rare myofibrillar content; the few myofibrils are mainly organized rudimentary and the nucleus is round and prominent.
- Purkinje-like cells (Fig. 4b,g) are classified into three different groups [66–68]. Structurally only one of these can be distinguished from atrial- and ventricular cardiomyocytes. This type of Purkinje-like cell is larger than atrial-/ventricular-like myocytes, possesses one or two round nuclei and shows a highly branched shape. The peripherally located myofibrils are aligned in the direction of tension forces. The Purkinje-like cells exhibit a gradual increase in the density of myofibrils and a decrease in cell size in transition to the atrial-/ventricular-like cells.
- The atrial-(Fig. 4c,h)/ventricular-(Fig. 4d,i) like cells are elongated (72–110 μm length), have an oval nucleus and well organized tension-orientated myofibrils, spanning the long axis of the cells.

Although all cardiac phenotypes simultaneously occur at each developmental stage in the EB, the percentage of the different cell types changes (from Ref. [61]): Early, pacemaker-like cardiomyocytes predominate in early EBs (7 + 4d, i.e. 11d in culture) while a higher percentage of atrial-/ventricular like cells is found in older EBs (17/21 days).

This morphological pattern of cardiomyocytes from EBs can also be distinguished by vital microscopy in early stage cells, pacemaker-like cells, atrial- and ventricular-like cells as exemplified in Fig. 4e–i. These cells have been recorded on videotape, prior to patch clamp recordings and characterized electrophysiologically (Fig. 4h, m, picture and action potential are not from the same cell). By this approach it is possible to correlate cellular morphology strongly with electrophysiological features of the different cardiac phenotypes as shown in Fig. 4k–n. Action potentials and ionic currents are measured on spontaneously beating cardiomyocytes using the patch clamp technique. While the atrial-like, Purkinje-like and ventricular-like cell types display a more stable and hyperpolarized resting potential (Fig. 4l, m, n), the early pacemaker-like cell is characterized by a relatively depolarized resting membrane potential (Fig. 4k). The independent phenotype classifica-

tion using morphological and electrophysiological criteria yielded nearly identical results. Thus developing cardiomyocytes can be selected by vital microscopy before the electrophysiological measurements.

7. Development of electrophysiological characteristics of ES cell derived cardiomyocytes

ES cell-derived cardiomyocytes express action potentials of sinusnodal, atrial and ventricular types: Differentiation of ES cell derived EB's allows for the first time to study in vitro all consequent stages of action potential development in mammalian cardiomyocytes (Fig. 5) [69,70]. At any given time during development cardiomyocytes with the different types of action potentials (early/pacemaker-, AV-, Purkinje-, atrial- and ventricular-like) can be found within the same EB. While cardiomyocytes of an early differentiation stage ('7 + 2 d' to '7 + 4 d') mostly reveal pacemaker-like action potentials, mainly three major types of action potentials can be found in cardiomyocytes of the terminal differentiation stage ('7 + 9 d' to '7 + 12 d'). Cells of atrial and ventricular phenotypes (elongated cells with orientated myofibrils) can be characterized by a stable resting potential of about −75 mV and by action potentials of high amplitude and upstroke velocity. Similar as described for adult myocardium [71,72], atrial action potentials differ from ventricular action potentials by a less pronounced plateau and by an acetylcholine-induced hyperpolarization. In cardiomyocytes of both phenotypes, tetrodotoxine (TTX), a selective blocker of voltage-dependent Na^+ channels, strongly reduces the upstroke velocity of action potentials. The 1,4-dihydropyridine-derived opener and blocker of L-type Ca^{2+} channels, BayK 8644 and isradipine, increases and decreases the plateau phase of action potentials, respectively. The third type of action potential measured in ES cell-derived cardiomyocytes (small, roundish cells with rare and disoriented myofibrils) shows all characteristics of sinusnodal pacemaker cells including the typical shape and the hormonal regulation (Fig. 5) (see also [73]). Chronotropic measurements demonstrated functional expression of adrenoceptors, cholinoceptors and L-type Ca^{2+} channels [44].

8. ES cell-derived cardiomyocytes express all major cardiac-specific ion channels

Undifferentiated stem cells exhibit no electrical activity. They are unable to generate action potentials and revealed only slight linear current voltage relations [74]. The various shapes of action potentials in ES cell-derived cardiomyocytes of different developmental stages are well correlated with the expression of specialized types of ion channels [70]. The expression of ionic currents and the corresponding action potentials measured in cardiomyocytes from different developmental stages are summarized in Fig. 5. While in cardiomyocytes of an early differentiation stage the primitive pacemaker action potentials are generated by only two main types of ion channels, i.e. voltage-dependent L-type Ca^{2+} channels (I_{Ca}) and transient K^+ channels ($I_{K,to}$), terminal differentiated cardiomyocytes express various additional types, including voltage-dependent Na^+ channels (I_{Na}), delayed outward rectifying K^+ channels (I_K), inward rectifying K^+ channels (I_{K1}), muscarinic acetylcholine-activated K^+ channels ($I_{K,Ach}$) and hyperpolarization-activated pacemaker channels (I_f). Ventricular- and atrial-like cardiomyocytes express I_{Na} and I_{K1} underlying the high upstroke velocity and the stable resting potentials, respectively [71,75]. Acetylcholine-induced hyperpolarization of atrial-like cells corresponds to the selective expression of $I_{K,ACh}$. Sinusnodal-like cells preferentially exhibit neither I_{Na} nor I_{K1}, but ion channels regulated by cardiotropic hormones, i.e., I_f, $I_{K,ACh}$ and I_{Ca} (see [73]). Most biophysical and

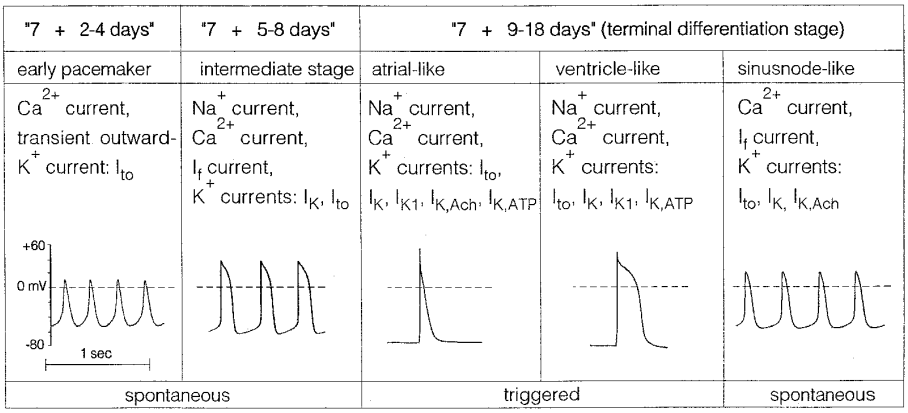

Fig. 5. Typical action potentials recorded in embryonic stem cell derived cardiomyocytes at different developmental stages in relation to ionic currents expressed in the cells. Both atrial and ventricular types of action potentials were elicited in current clamp mode by current pulse stimulation (from Ref. [70]).

pharmacological properties of the ionic currents of ES cell-derived cardiomyocytes are similar to those previously described for adult [24] or perinatal cardiomyocytes [76,19,77]. There are however some differences, which will be discussed below.

9. Ca^{2+} channels

The most prominent current component found throughout the entire differentiation period of ES-cell derived cardiomyocytes is the voltage-dependent I_{Ca}. The voltage dependence of I_{Ca} as well as parameters of steady-state inactivation are similar in cardiomyocytes of all differentiation stages. The whole-cell I_{Ca} exhibits all major biophysical properties and pharmacological characteristics inherent to the L-type Ca^{2+} current of the heart [72,78,79]. It is characterized by blockage with isradipine, gallopamil and Cd^{2+}. A specific antagonist of N-type Ca^{2+} current, ω-conotoxin, blocked I_{Ca} insignificantly. As reported for early stage embryonic, murine cardiomyocytes [22] T-type Ca^{2+} channels could not be detected in the ES-cell derived early stage cardiomyocytes. In some cardiomyocytes of intermediate and late stage T-type like Ca^{2+} currents with (i) negative threshold of activation, (ii) insensitivity to dihydropiridines, (iii) complete block by 50 μM Ni^{2+} were detected [70]; T-type Ca^{2+} currents were described in adult rat ventricle cardiomyocytes [123] and also the embryonic chick heart [124]. Bay K 8644, a specific opener of L-type Ca^{2+} channels, strongly stimulated the current (from 2 to 3 times) and shifted the maximum of the current–voltage curve to more negative potentials [70]. I_{Ca} was also stimulated by adrenaline or forskolin, a direct activator of adenylyl cyclase [80] suggesting a cAMP-dependent regulation for I_{Ca}. The regulation of I_{Ca} appears to differ between embryonal cardiomyocytes and adult ventricular myocytes. The development of signal transduction pathways for the regulation of ion channels preferentially has been studied at postnatal stages. In contrast to adult cardiomyocytes differences in the expression pattern of G-proteins and the activity of cAMP and cGMP can be found in postnatal rabbit and rat cardiomyocytes [81]. Moreover, a postnatal increase of I_{Ca} upon β-adrenergic stimulation was demonstrated [82], while there seems to be a postnatal decrease in the muscarinergic modulation of I_{Ca} [83]. Recent electrophysiological studies on embryonal murine cardiomyocytes and on ES-cell derived cardiomyocytes of early developmental stage provide a more detailed insight in developmental changes of the β-adrenergic and muscarinergic modulation of I_{Ca}. Ligand binding experiments have already proven expression of β-adrenoceptors as early as day 13 of murine gestation [84], but patch-clamp experiments on day 12–14 old murine embryonal cardiomyocytes have demonstrated the lack of I_{Ca} stimulation [85] upon isoproterenol application. Moreover, the cAMP analogue 8-bromo-cAMP did not stimulate I_{Ca}, indicating a limiting step in the β-adrenergic modulation of I_{Ca} downstream from the receptor and the G-protein. Cellular dialysis with the catalytic subunit of the cAMP-dependent protein kinase A (PKA) stimulated I_{Ca} and upon dialysis with the holoenzyme of PKA, I_{Ca} could be stimulated again by cAMP or forskolin. However, these cells still lacked the β-adrenergic modulation of I_{Ca} [85]. These and biochemical studies [86] support the idea of a reduced PKA activity in the embryonal heart. The insensitivity of I_{Ca} towards β-adrenergic modulation after dialysis with the holoenzyme of PKA points towards an additional defect in the signal transduction pathway. Hence, data from embryonal rats [87] show a lack of the functional coupling between G-protein and β-receptors in early gestation, suggesting a deficient receptor-G protein coupling. Studies in our laboratory have confirmed the lack of the β-adrenergic modulation of I_{Ca} for the young stage (7 + 2-4d) of ES cell-derived cardiomyocytes. In contrast, muscarinergic receptor stimulation strongly depresses basal I_{Ca} in the early stage cells. The muscarinergic depression of I_{Ca} is G-protein mediated, but not by a direct inhibition of the adenylylcyclase, as described for adult ventricular myocytes [88–90]. Preliminary results point towards an activation of the cGMP dependent phosphodiesterase II and a concomitant reduction in cAMP activity [91]. Further studies are needed to investigate the modulation of I_{Ca} in late stage cardiomyocytes.

The density of I_{Ca} significantly increases during cardiomyocyte differentiation in EBs up to 30 pA/pF [70]. This value is slightly higher than the reported current density of guinea pig and rat ventricular myocytes (26.5 pA/pF and 17–25 pA/pF, respectively) but lower than that of bovine ventricle (34–42 pA/pF) [79]. In chick embryonic cardiomyocytes a reduced or constant Ca^{2+} channel density has been reported [92,20] suggesting species dependent differences in the development.

10. Na^+ channels of low TTX sensitivity

I_{Na} could not be detected at the earliest differentiation stage ('7 + 2 d' to '7 + 3 d') but appears during further development [70]. While I_{Na} has been detected in rabbit sinus-node cells [125], ES cell derived sinus-node like cardiomyocytes do not express I_{Na}. Almost all terminally differentiated, ES derived cardiomyocytes express Na^+ currents. I_{Na} is maximally expressed in cells exhibiting atrial- and ventricular-like action potentials at the terminal differentiation stage [70]. A low TTX sensitivity of I_{Na} is a characteristic for the mammalian myocardium. TTX-block of I_{Na} in mouse cardiomyocytes was described by 1:1 binding with a K_d value of 1.4 μM [93]. The K_d value ranges from 1.5 to 2.5 μM for different mammalian cardiac preparations [94]. The whole-cell I_{Na} was further characterized by blockage with external Cd^{2+} (IC_{50} of near 0.5 mM). The Cd^{2+} sensitivity is an additional

specific property of cardiac Na^+ channels [95,96]. I_{Na} reverses closely to the calculated Na^+ equilibrium potential E_{Na} (46.6 mV). As previously described, positive to E_{Na} a slight inward-going rectification can be observed [97,98]. Major biophysical characteristics of I_{Na} including parameters of steady-state activation and inactivation prove to be similar to neonatal or adult mammalian heart cells [70].

The normalized conductance of I_{Na} at the intermediate differentiation stage is smaller than that of adult cardiac tissue. However, the density of I_{Na} apparently increases during cardiomyocyte differentiation from intermediate to terminal stage. From indirect estimations (by measurement of partially inactivated I_{Na}) the density of the current is more than 5 $\mu S/\mu F$. Interestingly, in some precursor cells of the intermediate stage the Na^+ channels are electrically inactivated due to the depolarized membrane potential of -40 mV to -60 mV (see also [99]). Thus the coexpression of inwardly rectifying K^+ channels (see below) leads to a lowering of the resting potential, to an increased availability of Na^+ channels and ultimately to the I_{Na}-triggered fast upstroke velocity of action potentials.

11. ES cell-derived cardiomyocytes express basic cardiac-specific K^+ currents

Three major K^+ currents, I_{to}, I_K and I_{K1} are present in ES cell-derived cardiomyocytes. I_{to} was expressed throughout the whole differentiation period in the spontaneously beating cells [70], similar to findings in murine embryonic cardiomyocytes [22]. Depolarization-activated I_{to} attains its maximum within about 10 ms and inactivates thereafter during 100–200 ms. The amplitude of I_{to} depends on the holding potential. The voltage of half inactivation varies in a range from -60 mV to -30 mV for cardiomyocytes of different ages with more negative values for cells of terminal differentiation as compared to those of early stages. The current density of I_{to} increases considerably in terminally differentiated cardiomyocytes as compared to the early differentiation stage. An augmentation in the density was reported for rat ventricular myocytes during postnatal development [76]. This results in shortening of ventricular action potentials, which is important for high beating rates of the heart. As demonstrated by the prolongation of action potential duration by 4-AP (about twice by 2 mM 4-AP), I_{to} plays a prominent role in the determination of the action potential duration in ES cell-derived cardiomyocytes.

I_K can be measured by membrane depolarization from a holding voltage of -45 mV where I_{to} is almost completely inactivated. I_K is stimulated by adrenaline and forskolin, suggesting a modulation by adrenoceptors through a cAMP-dependent pathway [100–102]. I_K can be mostly detected in cells of the intermediate and terminal differentiation stage.

The most prominent expression of the inwardly rectifying K^+ current (I_{K1}) is observed in cells with stable resting potential, i.e. in atrial- and ventricular cardiomyocytes. This current is evoked by hyperpolarizing voltage pulses and reveals a time-dependence which is more prominent at more negative potentials. I_{K1} exhibits the same properties as described for embryonal [19,103], perinatal [104,105] and adult [106,107] ventricular cardiomyocytes. I_{K1} is highly sensitive to external Ba^{2+} with 2 mM Ba^{2+} almost completely blocking the current. ES cell-derived cardiomyocytes reveal lower values of I_{K1} (usually less than 0.5 nA, by voltage pulse to -100 mV) as compared with those reported for adult cardiomyocytes (2–3 nA). An additional large increase of I_{K1} expression in mammals is found. This increase is suggested to be due to the appearance of the adult type of I_{K1}-channel of 42 pS, in addition to the embryonal type of 30 pS [104,105] or alternatively by a change in the open probability of the embryonal channel [126].

Furthermore, in cardiomyocytes of terminal differentiation the appearance of another type of K^+ current can be detected which progressively increases during prolonged (40–60 min) recordings of membrane currents in the whole-cell configuration. The K^+ selectivity of the current is confirmed by the measurement of its reversal potential which is close to the calculated E_K (-87 mV). The assumption that this current is due to openings of ATP-dependent K^+ channels is corroborated by the finding that its development is much faster at the onset (5–10 min) when the patch pipette solution is depleted of ATP. These results suggest the expression of ATP-dependent K^+ current ($I_{K,ATP}$) in ES cell-derived cardiomyocytes [108,109]. Recent experiments in our laboratory have demonstrated the activation of $I_{K,ATP}$ by the mitochondrial uncoupler 2,4-DNP in early and late stage cardiomyocytes (S. Viatchenko-Karpinski and B.K. Fleischmann, unpublished data). As also reported for murine, embryonic cardiomyocytes [22], the current density was similar in the early and late stage cells. This may indicate an important function of this channel during embryonic cardiac development.

At the terminal differentiation stage, ES cell-derived cardiomyocytes (about 30% of all cells assayed) of the atrial and sinusnodal types selectively express inward rectifying K^+-currents ($I_{K,Ach}$) activated by the cholinoceptor agonist carbachol. Under carbachol, $I_{K,Ach}$ is seen as an inward rectifying current component depending on extracellular K^+ concentration. After its activation, $I_{K,Ach}$ is almost completely suppressed by subsequent addition of 20 μM atropin, suggesting the involvement of muscarinic receptors [110]. Under conditions of equimolar K^+ solutions we have recently detected $I_{K,Ach}$ in more than 50% of early stage cells (S. Viatchenko-Karpinski and B.K. Fleischmann, unpublished data). The current density is low and the functional importance is questionable, because application of the muscarinergic agonist carbachol causes only in about 20% of the early stage cells a hyperpolariza-

tion of the resting membrane potential (Q. Ji and B.K. Fleischmann, unpublished data).

12. Pacemaker (I_f)-current

Another current component important for hormonal regulation of pacemaker action potentials is the hyperpolarization-activated current (I_f) current [111,112]. Expression of I_f could be detected in early-, pacemaker- and Purkinje-like cells, but not in atrial- and ventricular-like cells [70]. This is in contrast to findings in chick atrial and ventricular cardiomyocytes [127] and to a recent report, where I_f was also found in adult canine ventricular cardiomyocytes at very negative potentials [128]. The amplitude of I_f varied in individual ES-cell derived cardiomyocytes between 50 and 500 pA (measured at hyperpolarizing voltage steps from -35 mV to -100 mV). In the terminally differentiated pacemaker-like cells, current densities up to 2 nA were observed. All cells expressing I_f generate spontaneous pacemaker action potentials.

The shape of the hyperpolarization-activated I_f and its steady-state activation curve in cardiomyocytes of terminally differentiated pacemaker like cells proves to be similar to those previously found in sinusnodal cells or Purkinje fibres from adult hearts (for review see [113], for embryonic chick heart see [114,115]). The I_f activation curve measured from a holding potential of -35 mV extends over the range from -40 mV to -100 mV [70]. The reversal potential obtained from the current–voltage relationship can be found at about -27 mV. The current almost completely disappears in 'cation free'-medium suggesting that I_f channels are predominantly permeable for cations. I_f can be blocked by 2 mM Cs^+. Adrenaline and carbachol stimulate and inhibit I_f shifting the current activation curve to positive and negative potentials, respectively. In addition, forskolin modifies I_f similar as adrenaline suggesting a cAMP-dependent regulation of the current [70].

13. Limitations of the ES-cell preparation

While in the late stage cells, cardiomyocytes can be recognized by their typical myofibrillar content under the light microscope prior to measurements by the patch clamp technique, cells of the earliest stage cannot easily be distinguished from other cell types by morphological criteria (small, roundish shape). For this reason, electrophysiological measurements on early stage cells have to be exclusively performed on spontaneously beating cells. This type of cell selection bears the risk of overlooking an early cardiomyocyte population, which does not yet contract. Moreover, as described above, the M-band and T-tubulus-formation is not finalized, indicating that cardiomyogenesis only reaches a perinatal-like stage. Differentiation protocols have to be standardized in order to exclude clonal diversity. Some differences of ion channel expression and biophysical characteristics between the ES cell derived and fetal, mammalian cardiomyocytes have been also noticed (see chapters above).

14. Conclusions and future perspectives

Taken together, the data reviewed suggest that pluripotent embryonic stem cells cultivated within EBs reproduce cardiomyocyte development from primitive precursor cells to highly specialized phenotypes of the cardiac tissue. The differentiation of cardiomyocytes in EBs represents a process of developmentally controlled gene expression [116–118], increasing myofibrillar organization, changing cellular shape and size as well as electrophysiological properties. It is demonstrated that most cardiac-specific ion currents, L-type I_{Ca}, I_{Na}, I_{to}, I_K, I_{K1}, $I_{K,Ach}$, $I_{K,ATP}$ and I_f are expressed in cardiomyocytes developed in vitro from pluripotent ES cells. Most of the biophysical and pharmacological properties of ion currents were similar to those previously described for adult cardiomyocytes or neonatal mammalian heart cells [19,21,76,105,104, 119,120].

The ES-cell derived cardiomyocyte differentiation system has the following advantages: (i) cardiomyocytes develop among cells of all 3 germlayers in contrast to monocultures; (ii) nearly two-dimensional growth allows microscopical observation during development; (iii) morphological and electrophysiological characterization of the same cell; (iv) easy access to very early stage cardiomyocytes.

Furthermore, this cell model is valuable for more detailed studies on commitment and differentiation of cardiomyocytes, on the role of growth factors, extracellular matrix components and connexins, on cardiac myogenesis, as well as on pharmacological and toxicological effects on morphology, gene expression, cardiac-specific ionic currents and action potentials. It may further provide a unique model to analyze the quantitative expression of ion channels and the corresponding structural changes during the cardiac development which may reveal new insights into inborn heart diseases (see review, [121]). A first attempt for studying the role of the extracellular matrix (β1-integrins) has been made already [58], finding that the lack of integrins significantly influence cardiac development, in particular the expression of ion channels as well as myofibrillar proteins. The absence of integrins leads to a retarded differentiation of cardiomyocytes.

Simultaneous quantitative analysis of channel expression [122] and ultrastructure in ES cell-derived cardiomyocytes as well as temporally and spatially controlled gene expression may offer for the first time the possibility to study pathophysiological phenomena. For example, an abnormal development of ionic channels may lead to an

electrical instability of the cardiomyocyte and consequently to arrhythmias. A limitation for many studies is the identification of cardiomyocytes, especially of the early stage. This appears particularly critical for molecular biological and biochemical studies, where a pure population of cardiomyocytes is needed. This may help to address such critical questions as time point of differentiation into the cardiac lineage and regulation of gene expression. Recently Metzger et al. have established an ES-cell line, where lacZ expression was under the control of the cardiac specific promoter human cardiac α-actin [129]. This allowed also a vital stain approach, in order to investigate early stage cardiomyocytes functionally. In the future, this approach may be further improved by the establishment of stably transfected ES-cell lines, where instead of lacZ in vivo reporter genes are under control of very early, cardiac specific promoters.

In the future the ES-cell differentiation model may prove also helpful for the investigation of the development of other cell types.

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