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Cardiovascular Research 58 (2003) 278–291

Cardiovascular Research

www.elsevier.com/locate/cardiores

Review

Cardiac specific differentiation of mouse embryonic stem cells

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Received 12 November 2002; accepted 9 January 2003

Abstract

Embryonic stem (ES) cells may represent an alternative source of functionally intact cardiomyocytes for the causal treatment of cardiovascular diseases. However, this requires cardiac-specific differentiation of stem cells and the selection of pure lineages consisting of early embryonic cardiomyocytes. Therefore, an understanding of the basic mechanisms of heart development is essential for selective differentiation of embryonic stem cells into cardiac cells. The development of cardiac cells from embryonic stem cells is regulated by several soluble factors and signalling molecules together with cardiac specific transcription factors such as the zinc-finger GATA proteins and Nkx-2.5. GATA-4 and Nkx-2.5 seem to be essential for heart development. The use of enhanced green fluorescent protein (EGFP) under the control of cardiac-specific promoters in combination with the ES cell system has allowed for the functional characterisation of cardiac precursor cells. Embryonic stem cell-derived cardiomyocytes developmentally express similar cardiac-specific proteins, ion channels and signalling molecules to that of adult cardiomyocytes. Furthermore, identification of growth factors and signalling molecules under cell culture conditions is crucial for the selective cardiac differentiation of embryonic stem cells. Therefore, serum-free culture conditions have to be established in order to examine the influence of different growth factors and signalling molecules on cardiac development and/or formation from ES cells. Although significant progress has been made in generating cardiac cell lineage by the combination of genetically manipulative methods with selective culture conditions for cell transplantation therapy, one of the remaining future challenges for transplantation in humans is the immunological rejection of the engrafted cardiomyocytes.

Keywords: Embryonic stem cells; Cardiomyocytes; Signalling molecules; Growth factors

1. Introduction

Heart failure represents one of the most frequent cardiovascular diseases in the world that develops as a result of the inability of the heart to pump enough blood to maintain physiological functions. Heart failure is characterised by the loss of functional cardiomyocytes (also known as cardiomyopathy). Because adult cardiomyocytes are unable to regenerate, heart transplantation is the only effective treatment of patients suffering from severe heart failure. In the last decade several animal studies demonstrated that transplantation of isolated cardiomyocytes (also known as cardiomyoplasty) might offer an alternative approach for the treatment of severe heart failure. In this context, several animal studies demonstrate a successful engraftment of cardiac myocytes into the adult heart [1–5]. However, the limiting factor for a comprehensive application of cell therapy for treatment of cardiovascular diseases is still the insufficient number of donor cells. Since treatment of heart failure in humans by embryonic heart cells from aborted fetuses is prohibited ethically, embryonic stem (ES) cells could be an alternative source of functionally intact cardiomyocytes for the causal treatment of cardiovascular diseases. Therefore, many efforts have been made to develop strategies for differentiation of early cardiomyocytes from ES cells and for lineage selection. ES cells isolated from the inner cell mass of the early mammalian blastocyst-stage embryo are pluripotent [6–8].

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Time for primary review 29 days.

0008-6363/03/$ – see front matter
doi:10.1016/S0008-6363(03)00248-7

In the presence of relatively high concentrations of leukemia inhibitory factor (LIF), murine and human ES cells remain undifferentiated [6,9] when they are cultured on embryonic fibroblast feeder layers. In the absence of LIF, ES cells spontaneously differentiate into multicellular ES cell aggregates (ESCAs), which resemble early post-implantation embryos. In general, it is believed that formation of ESCAs initiates signalling and spontaneous differentiation of ES cells to the three embryonic germ layers, the ectoderm, mesoderm and endoderm (for review see [6–8,10]). In this context, it has been demonstrated that mouse ES cells are able to differentiate in vitro into cardiomyocytes, hematopoietic progenitors, yolk sac, skeletal myocytes, smooth muscle cells, adipocytes, chondrocytes, endothelial cells, melanocytes, neurones, glia, pancreatic islet cells, and primitive endoderm (for review see [6]). The present review is focused on the differentiation of ES cells to cardiac cells. Nevertheless, understanding the basic mechanisms of heart development as well as the signal transduction pathways of several transcription factors promoting cardiac differentiation is a prerequisite for selective differentiation of ES cells to pure cardiac cell lineages.

2. Basic mechanisms of heart development

Basic mechanisms of heart development (cardiogenesis) are conserved between vertebrates and invertebrates. Identification of soluble growth factors, transcription factors and signalling cascades capable of priming cardiogenesis is a crucial issue for the in vitro development of cardiomyocytes from ES cells. The heart is the first mesoderm-derived functional embryonic organ that is developed after gastrulation (Fig. 1). The process of heart development involves proliferation and differentiation as well as organisation of tissue into the specific anatomical structure. In general the sum of stimulatory and inhibitory signals determine the medial-lateral borders of the heart-forming region (Fig. 1) [11,12]. There is accumulating evidence that development of uncommitted mesodermal precardiac cells to early cardiac cells is regulated by stimulatory signals secreted by the anterior primitive endoderm [13] (Fig. 1) [14–17]. In addition, various signals from the lateral regions of the embryo are also essential for heart formation [16,17]. The bone morphogenetic protein (BMP-2) plays a central role in the induction of heart formation in vertebrate embryos [17–19]. It is well known that factors from the anterior endoderm in combination with BMP signals in the anterior lateral mesoderm promote heart formation [11] (Fig. 1). Inhibitory signals mediated by the Wnt proteins, secreted by the neuronal tube, suppress cardiogenesis in the adjacent mesoderm [8] (Fig. 1); accordingly, Wnt antagonists such as Crescent [12] and Dkk1 [20] appear to induce cardiogenesis.

2.1. BMP-2 signalling and cardiogenesis

BMPs belong to the transforming growth factor β (TGF-β) superfamily and are expressed in lateral endoderm and ectoderm. Expression of cardiac-specific proteins by BMP-2 appears to be mediated by the transcription factors GATA-4 and Nkx-2.5. In this context, application of BMP-2 in vivo induced ectopic expression of the cardiac transcription factors Nkx-2.5, transcription factors of the GATA family (GATA-4, GATA-5, GATA-6) [21–25] as well as cardiac-specific proteins such as ventricular myosin

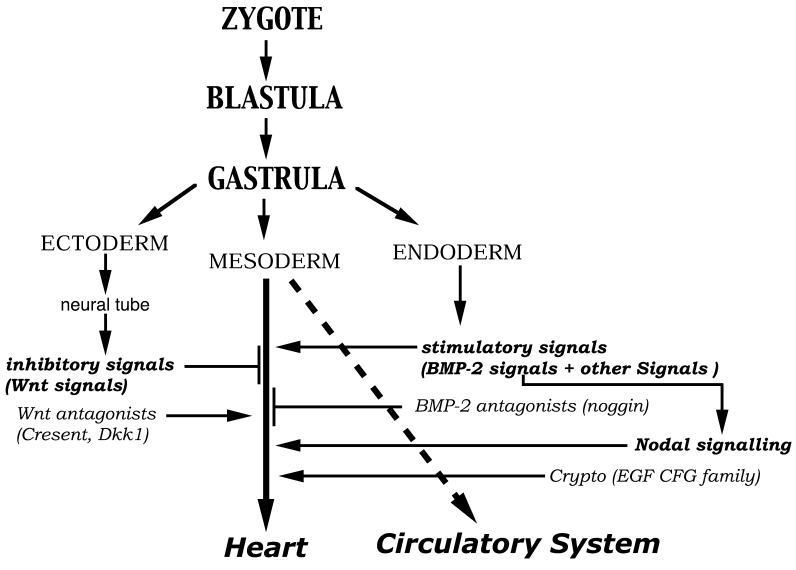

Fig. 1. Regulation of heart development from mesodermal germ layer by different signal transduction pathways (for more details see text).

heavy chain (vMHC) [11,17,18]. Regulation of the GATA proteins occurs by other transcription factors and by transcriptional coactivators and corepressors. Nkx2.5 is a coactivator of GATA-4 that acts via association with the C-terminal zinc finger of GATA-4 thus eliciting the transcription of cardiac-restricted genes [26–29]. Interestingly, mutations in Nkx-2.5 are involved in congenital human heart diseases [30] and impair normal heart architecture by affecting cardiac developmental pathways in patients with heart diseases [31,32]. Friend of GATA-2 (FOG-2) that is expressed in adult heart is also involved in the development of heart. Similar to Nkx-2.5, FOG-2 physically associates with the N-terminal zinc finger of GATA-4 in vitro and in vivo. Physical interactions of GATA-4 with FOG-2 specifically modulate the transcriptional activity of GATA-4 suggesting that FOG-2 also modulates GATA-4 function during cardiac development [25]. Six GATA proteins (GATA1–6) have been identified containing zinc finger DNA-binding domains, with the consensus binding motif, WGATAR; binding is also possible to related sequences such as CGATGG and AGATTA [24,25,33]. GATA-4 promotes cardiac muscle development and regulates the expression of several cardiac specific genes including α-myosin heavy chain (α-MHC), cardiac troponin C and atrial natriuretic peptide [25,32,34]. It has been shown that disruption of the GATA-4 gene in mice leads to early embryonic lethality because of specific defects in ventral heart tube formation [25,35,36]. Furthermore, administration of soluble BMP-2 or BMP-4 to explant cultures induces full cardiac differentiation in stage 5–7 anterior medial mesoderm, a tissue that is normally not cardiogenic [17]. However, when the adjacent neural tube and notochord is included in these explants, BMP-2 administration only induces Nkx-2.5 and fails to induce expression of either GATA-4 or vMHC [17]. Competence to undergo cardiogenesis in response to BMPs is restricted to mesoderm located in the anterior regions of gastrula- to neurula-stage embryos.

The heart-promoting activities of the anterior endoderm and BMPs are antagonised by repressive signals from the axial tissues that block cardiogenesis in the anterior paraxial mesoderm [11]. The secreted protein noggin, which binds to BMPs and antagonises BMP activity, completely inhibits differentiation of the precardiac mesoderm, indicating that BMP activity is required for myocardial differentiation in this tissue [17].

Recently, it has been shown that BMP-2 signalling in chick embryo mesoderm is essential for the rapid activation of nodal signalling and its target genes Pitx2 and Nkx3.2 [37,38]. Several mutation studies demonstrated that nodal, a TGF-β related molecule [39], is involved in the formation of mesoderm [40,41] and is required for heart development in mouse embryo [42]. Furthermore, BMP signalling positively regulated the expression of the Snail-related gene and the Cfc in chick embryo mesoderm [37,38]. The zinc finger protein Snail is essential for the formation of mesoderm [43], while Cfc proteins belong to the EGF–CFC family of proteins that play an important role in general developmental processes (for review see [44]). EGC–CFC proteins act as cofactors for nodal-related signals [45]. Several studies suggest that Cripto, another protein of the EGF–CFC family containing a Cfc domain is involved in heart development [44]. More recently, stimulation of ESCAs generated from ES cells with BMP-2 and TGF-β resulted in increased cardiac differentiation with a significant increase in beating areas and enhanced myofibrillogenesis [46]. In the presence of postmitotic cardiomyocytes, stem cells differentiate into ventricular myocytes and beat in synchrony with host cells. This process was significantly enhanced by TGF-β or BMP-2 [46]. In vitro, disruption of the TGF-β/BMP signalling pathways by noggin prevented differentiation of ES cells [46]. TGF-β itself is an autocrine growth factor that is normally implicated in the development of tumour diseases. Recently, several members of the TGF-β superfamily were discovered to act as morphogens thereby playing an important role in embryogenesis. In Drosophila the TGF-β homologue dpp encoded by dpp is required for mesoderm formation and cardiogenesis [47–50]. Binding of TGF-β to its serine/threonine kinase receptor activates signalling of TGF-β via phosphorylation, stabilisation and activation of the heterotetrameric TGF-β receptor complex (for review see [51,52]) (Fig. 2). This results in phosphorylation and subsequent activation of transcription factors belonging to the Smad family. The Smad proteins are subdivided into receptor-regulated Smads (R-Smads) which are phosphorylated by the TGF-β receptor complex and common Smads (Co-Smads) which hetero-oligomerise with the R-Smads and finally the inhibitory Smads. Phosphorylated R-Smads are able to migrate to the nucleus and to hetero-oligomerise with the Co-Smad. In the nucleus, the hetero-oligomeric complex binds to DNA in a site-specific fashion and interacts with a variety of transcription factors, coactivators and corepressors, thereby inducing the expression of TGF-β-responsive genes. Although stimulation of the Smad pathway is the major pathway, activation of the MAP kinase pathway, including the extracellular response kinases 1 and 2 (ERK1/2) and the c-jun N-terminal kinases (JNKs) may also be mediated by TGF-β in some cell types. In contrast to the Smad pathway that is probably involved in developmental processes, activation of ERK1/2 and JNKs appears to be required for motility and epithelial–mesenchymal transformation induced by TGF-β [51,52].

2.2. Growth factors and cardiogenesis

Insulin-like growth factor I (IGF I) has been shown to be essential for normal embryonic growth in mice [53] and for the formation of a functional heart (for review see [54]). Activation of the insulin receptor (IR)/IGFR family by insulin and IGF results in the tyrosine phosphorylation

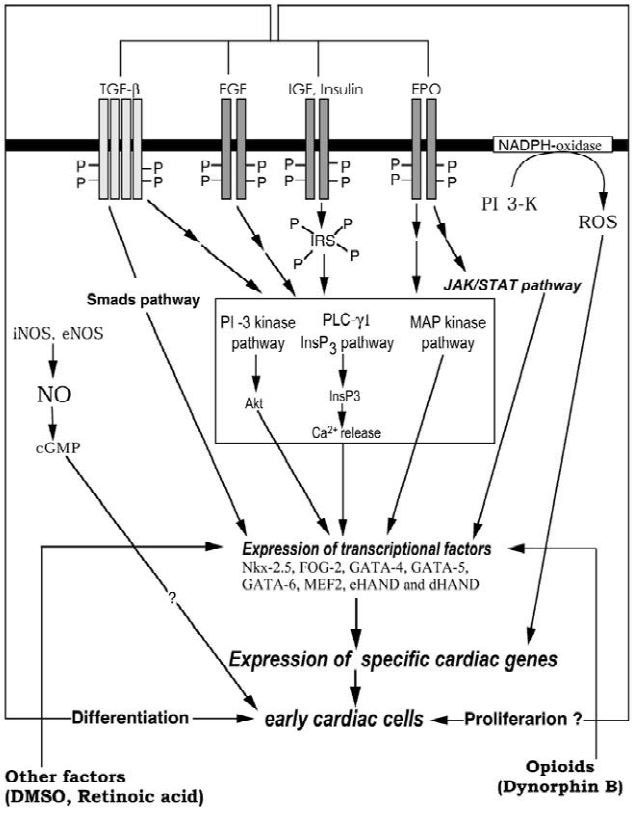

Fig. 2. Possible intracellular transduction pathways promoting generation of cardiomyocytes by different growth factors, other factors such as D.M.S.O., retinoic acid and opioids such as dynorphin B (for more details see text) as well as by different signal molecules such as NO and R.O.S. Please note that it is not clear whether growth factors induce both differentiation and proliferation of early cardiomyocytes.

of a variety of intracellular insulin receptor substrates (IRSs) (for review see [55]). Phosphorylated IRSs activate phosphoinositide 3-kinase (PI 3-K) converting the plasma membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate [55,56]. Phosphorylated IRSs are also able to phosphorylate and thus activate other kinases such as the phospholipase C-γ1 (PLC-γ1), Akt/protein kinase B (PKB), $p70^{rsk}$ and protein kinase C (PKC) [55]. Activation of PLC-γ1 results in hydrolysis of PIP_2 to diacylglycerol (DG) and $InsP_3$ that releases Ca^{2+} from endoplasmic reticulum. A second signalling pathway involves activation of the small G-protein Ras, which in turn activates the protein serine kinase Raf and the MAPK cascade including the ERK1/2 [55] (Fig. 2). Using ESCAs we provided evidence that signal transduction via the PI-3-kinase pathway might be essential for mammalian early cardiomyocytes development [57,58] (for more details see Section 2.6).

Fibroblast growth factor (FGF) signalling seems to be also involved in heart formation of vertebrates. Interestingly, mutation of FGF in zebrafish, normally expressed in the cardiogenic mesoderm, results in a decreased expression of cardiac markers [59]. Moreover, the early development of avian pericardial mesoderm has been found to be regulated by FGF-2, insulin and insulin-like growth factors [60]. To date, 20 FGFs have been identified belonging to the FGF family of small polypeptide growth factors (for review see [61]). Synergistic activation of the FGF receptors with heparan-like glycosaminoglycans results in dimerisation and tyrosine phosphorylation of the FGF receptors following tyrosine phosphorylation of PLC-γ1 and Src tyrosine kinases. Moreover, activation of the MAP kinase cascades occurs via the Ras/Raf pathway (Fig. 2) [61].

Studies on mice lacking erythropoietin $(EPO)^{-/-}$ and erythropoietin receptor $(EPOR)^{-/-}$ suggest that EPO and EPOR are implicated in heart development [62]. This has been demonstrated by the observations that embryonic heart development in $EPO^{-/-}$ and $EPOR^{-/-}$ animals suffer from ventricular hyperplasia and defects in the interventricular septum. Erythropoietin (EPO) is a growth factor promoting formation of erythrocytes and proliferation and/or differentiation of megakaryocytes [62–66]. EPORs are also expressed in nonhematopoietic cells such as umbilical cord and placental endothelial cells as well as in neurons [62]. EPO also induces proliferation of endothelial cells [62–67]. The EPO receptor belongs to the cytokine receptor superfamily. Similar to the IL-6 receptor, binding of EPO results in homodimerisation and autophosphorylation of the EPOR following stimulation of the Janus kinases/signal transducers and activators of transcription (JAK/STAT) pathway, the PI 3-K and the MAPK pathway (for review [68,69]) (Fig. 2). $EPO^{-/-}$ and $EPOR^{-/-}$ mice further demonstrate that EPO is involved in control of proliferation, survival and irreversible terminal differentiation of erythroid progenitors [62]. Cardiac abnormalities in $EPO^{-/-}$ and $EPOR^{+/-}$ lacking mice are likely due to a reduction of proliferation, specific to the heart, since proliferation in other organs including brain in EPOR null animals is indistinguishable from that of wild mice [62]. After creation of chimeric animals by injecting $EPOR^{-/-}$ in the wild-type blastocysts it has been shown that EPOR is essential for the normal structure and development of the heart.

2.3. Other factors promoting cardiogenesis

It is well known that retinoic acid accelerates differentiation of ES cells to the cardiac lineage [70]. Dimethylsulfoxide (DMSO) promotes the differentiation of ES cells [32] as well as of the P19 embryonal carcinoma cell line to embryonic cardiac cells [71], and induces the expression of cardiac specific transcription factors GATA-4 and Nkx-2.5 in P19 cells [72] (Fig. 2). Furthermore, inhibition of GATA-4 inhibited the DMSO-induced cardiogenesis whereas transfection of Nkx-2.5 to ES cells in the absence of DMSO led to the appearance of myocardial cells [32,72]. Interestingly, there is increasing evidence that

opioid peptides are regulators of cardiogenesis: adult cardiac myocytes express the prodynorphin gene and are able to synthesise and secrete dynorphin B, a natural κ-opioid. P19 cells and murine ES cells synthesise and secrete dynorphin B, a biologically active end product of the prodynorphin gene (Fig. 2) [32]. DMSO-induced expression of GATA-4 and Nkx-2.5 gene expression is preceded by a marked increase in prodynorphin gene expression and dynorphin B synthesis and secretion [32]. In the absence of DMSO, dynorphin B triggered GATA-4 and Nkx-2.5 gene expression and led to the appearance of both α−MHC and MLC-2v transcripts [32]. Opioid receptor antagonists blocked DMSO-induced cardiogenesis suggesting the involvement of an autocrine opioid gene in the development of heart [32].

2.4. Serum response factor and cardiogenesis

Activation of serum response factor (SRF) mediates growth factor-stimulated transcription of immediate-early genes (IEGs). Expression of IEGs appears to be regulated by SRF and Elk-1 protein. Both proteins bind to serum response elements (SREs) in promotors of IEGs. Phosphorylation of Elk-1 occurs by ERK1/2 activity stimulated by serum and different growth factors, resulting in a transcription of IEGs [73] (Fig. 3). Recently, it has been demonstrated that serum response factor (SRF) is essential for murine embryogenesis [74]. In this context, mouse lacking Srf ($Srf^{-/-}$) stop developing at the onset of gastrulation, lacking detectable mesoderm and mesodermal marker genes such as BMP-2. Furthermore, the ability of $Srf^{-/-}$ ES cells to differentiate in vitro to mesodermal cells was impaired. However, impairment could be modulated by external cell-independent factors such as retinoic acid that induced expression of mesodermal marker genes [74]. Mesodermal marker gene expression such as Brachyury is also observed when SRF is expressed in $Srf^{-/-}$ ES cells. These findings suggest that SRF contributes to mesodermal gene expression of ES cells and that $Srf^{-/-}$ ES cells possess a nonautonomous defect in differentiation toward mesoderm [74].

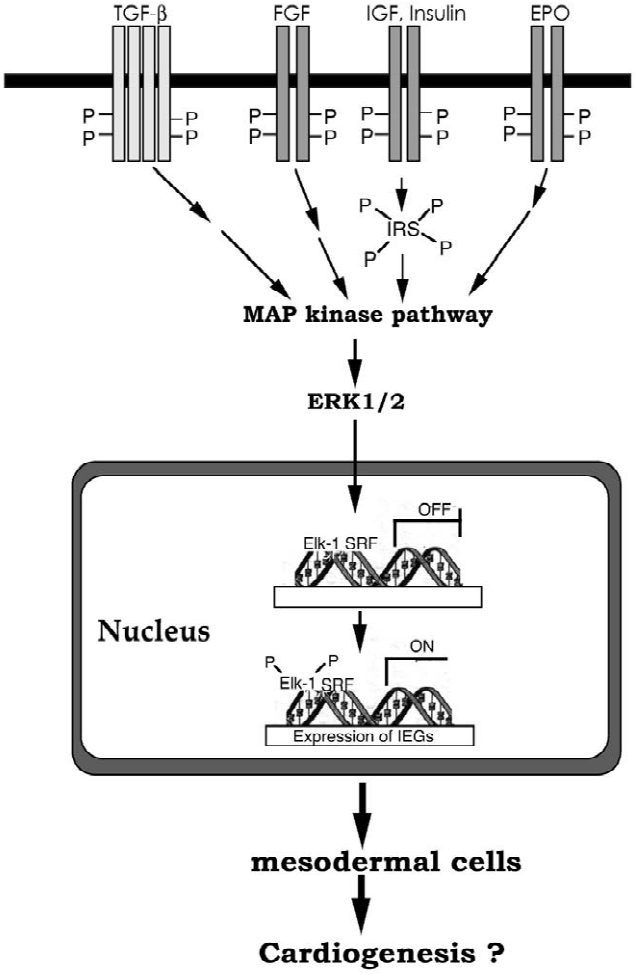

Fig. 3. Activation of SRF via the ERK1/2 pathway stimulated by different growth factors. Activation of SRF seems to be essential for mesoderm formation. However, it is not clear whether the SRF-induced mesodermal cells are able to differentiate to cardiac cells (for more details see text).

2.5. Wnt signalling and cardiogenesis

Wnts are secreted signalling glycoproteins that regulate key developmental processes in Drosophila (Wingless, homologues to Wnts) and vertebrates [75–77]. Wnts belong to a family of cysteine-rich glycosylated ligands (more than 16 mammalian family members) that mediate cell–cell communication in diverse developmental, cell growth and survival processes. The loss or inappropriate activation of Wnt expression has been shown to alter cells fates, morphogenesis as well as mitogenesis (for review see [78]). After binding of Wnt to its receptor complex consisting of a member of the Frizzled family of seven transmembrane proteins and the LDL-receptor related proteins (LRP5 and LRP6), a signal transduction cascade is stimulated resulting in the hyperphosphorylation of a cytoplasmic protein known as Dsh (Fig. 4) [78–81]. Activated Dsh inhibits the glycogen synthase kinase-β (GSK-β), a serine/threonine kinase. Inactivation of GSK-β leads to an accumulation of cytoplasmic β-catenin (Armadillo in Drosophila) and its translocation to the nucleus. β-Catenin interacts with the T-cell factor (TCF)/Lef transcription factor resulting in the expression of developmental target genes inhibiting cardiac development in vertebrates (also known as the canonical Wnt-catenin-TCF/Lef signaling pathway) (Fig. 4). In the absence of Wnt signaling GSK-β targets β-catenin for degradation, thereby inhibiting expression of target developmental genes (Fig. 4). Inhibitory signals that block heart formation in anterior paraxial mesoderm include Wnt family members expressed in dorsal neural tube (Wnt-1 and Wnt-3a) and anti-BMPs expressed in the axial tissues (i.e. noggin in the notochord) (Fig. 1). Ectopic Wnt signals can repress heart formation

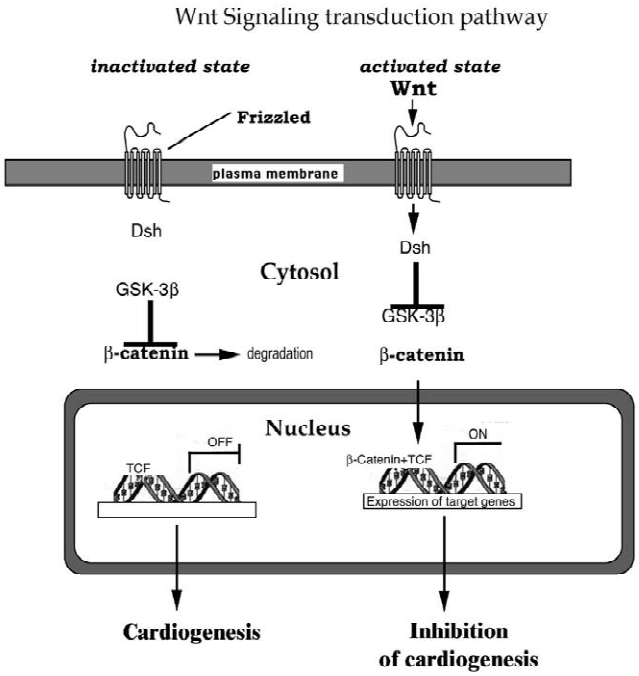

Fig. 4. Wnt signal transduction pathway and inhibition of the cardiogenesis (for more details see text).

from anterior mesoderm in vitro and in vivo. In this context, signals from the neural tube that blocks cardiogenesis in mesoderm of stage 8–9 chick embryos can be mimicked by ectopic expression of either Wnt-3a or Wnt-1h of which are expressed in the dorsal neural tube [11,12]. Repression can be overcome by ectopic expression of Wnt antagonist [11]. However, it has been demonstrated that noncanonical Wnt signalling mediated by Wnt11 promotes early avian mesoderm and cardiogenesis [82,83] and Wnt11 is required for heart formation in Xenopus embryos [84]. There in increasing evidence that Crescent, a Wnt-8 antagonist promotes cardiogenesis [12]. Crescent is a Frizzled-related protein that is expressed in anterior endoderm during gastrulation [16]. Dickkopf1 (Dkk1) is another class of Wnt antagonists that inhibit both Wnt-8 and Wnt-3a. Dkk1 induces heart-specific gene expression in posterior lateral plate mesoderm [20,85]. More recently, it has been shown that the Dkk-1 is upregulated by several genotoxic stimuli and overexpression of Dkk1 enhances BMP-mediated apoptosis in vertebrates [86]. Furthermore, expression of Dkk1 is regulated by c-Jun [86], a MAP kinase activated by different stress stimuli [87]. These results suggest that stress stimuli may promote selective cardiac development via upregulation of Dkk1 and enhancement of the apoptosis of noncardiac cells.

2.6. Role of PI 3-kinase and reactive oxygen species in cardiomyogenesis of ES cells

The intracellular redox state is characterised by the balance of oxidant production and the antioxidant capacity of the cell based on a variety of antioxidant enzymes such as superoxide dismutase (which reduces $O_2^{-}\cdot$ to H_2O_2), catalase, and glutathione peroxidase (which reduce H_2O_2 to H_2O). Apart from antioxidant enzymes, all cells contain a variety of reducing substances, e.g. the vitamins C, A, E, lipoate, thiols, urate, ubiquinone, glutathione (GSH), thioredoxin, and glutaredoxin which efficiently scavenge reactive oxygen species (ROS) and together with the antioxidant enzymes balance the ratio of the concentration of oxidizing equivalents to the concentration of reducing equivalent [88]. On the other hand it is a well known feature that cells are capable of generating ROS which are involved in the induction and maintenance of signal transduction pathways implicated in cell growth and differentiation. Among others cardiac cells have been shown to elicit a small oxidative burst generating low concentrations of ROS when they are stimulated by cytokines, growth factors and hormones that confer hypertrophic cell growth, e.g. epinephrine and endothelin [89] as well as tumor necrosis factor-α (TNF-α) and angiotensin II (ANGII) [90]. This led to the assumption that the initiation and/or proper functioning of several signal transduction pathways resulting in hypertrophic cell growth rely on the action of ROS as signalling molecules which may act on different levels in the signal transduction cascade [91]. This is self-evident, as ROS ideally fulfil the prerequisites for intracellular signalling molecules since they are rapidly generated, highly diffusible, easily degraded and ubiquitously present in all cell types. In the heart oxidative stress has been traditionally implicated in cell death following myocardial infarction or reperfusion injury. Oxidative stress in cardiac cells may arise from ROS released from mitochondria, or generated through the activity of xanthine oxidase, and the phagocytic nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase. A nonphagocytic NAD(P)H oxidase has been demonstrated to be involved in the development of left ventricular hypertrophy and heart failure progression [92] supporting the notion that activation of the nonphagocytic NAD(P)H oxidase in response to neurohormones is mediating cardiac myocyte hypertrophy. During cardiac hypertrophy parts of the genetic program of foetal life are re-expressed, which implies that foetal signalling pathways are reactivated. This may also hold true for signalling pathways involving ROS in the regulation of cardiac growth and differentiation. Recently, we demonstrated that inhibitors of PI 3-K abolished cardiac commitment in ES cells [57,58] (Fig. 5A). PI 3-K belongs to a broad family of kinase isoforms that have emerged as crucial regulators of many cell functions including cell division, cell migration and cell secretion. In this context, we demonstrated that a specific inhibitor of PI 3-K, LY294002, induced a reduction of α-actinin-stained beating cardiomyocytes in murine ESCAs (Fig. 5A). In parallel we observed a strong decrease in the number of ESCAs containing area(s) with beating cardiomyocytes (Fig. 5A). Since formation of

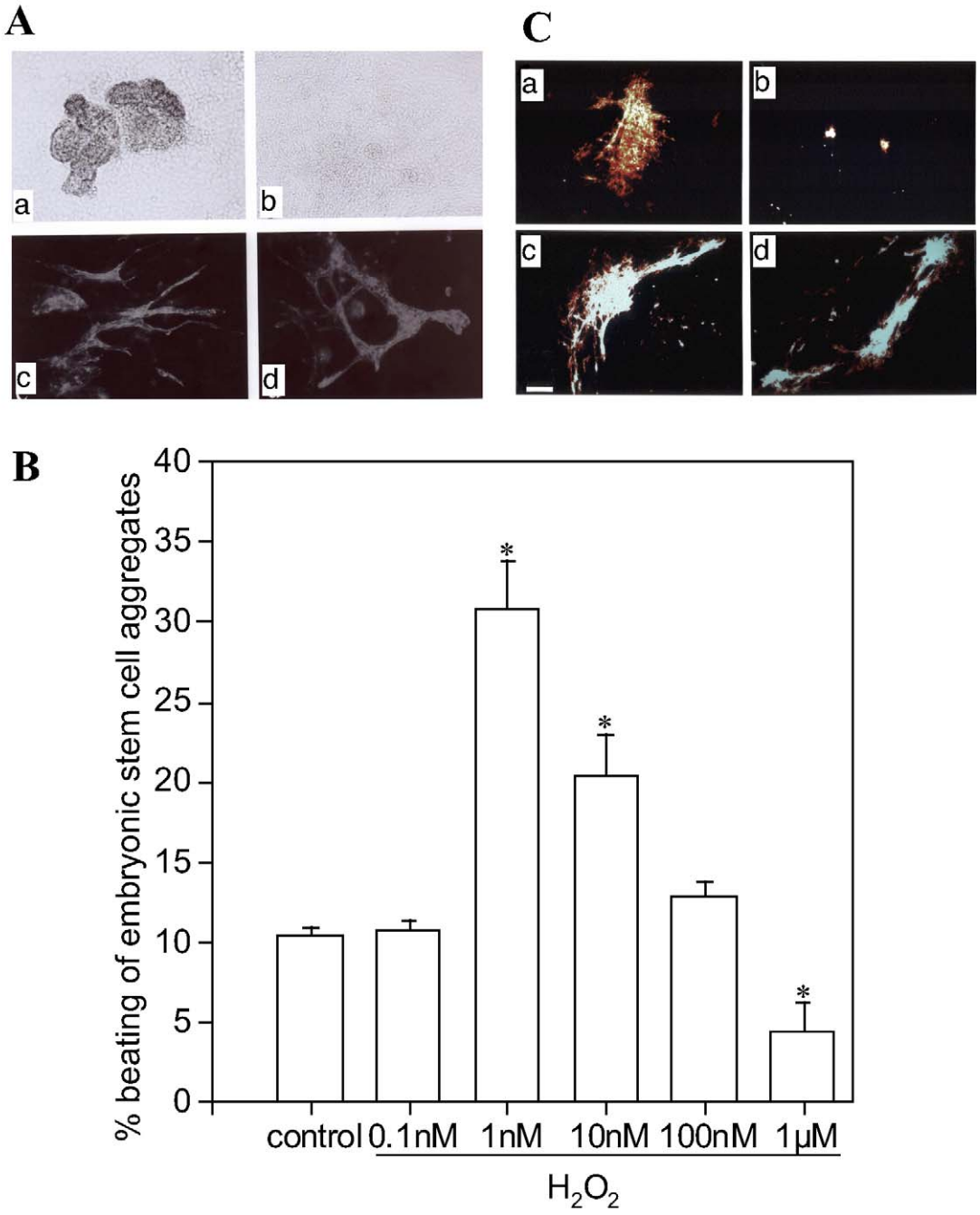

Fig. 5. Effects of LY294002 and H_2O_2 on cardiomyocyte differentiation of murine ES cells. (A) ESCAs were exposed from days 3 to 7 to 0.1% DMSO as a control (a,c) or to 50 μM LY294002, a PI 3-K inhibitor (b,d). Indirect co-immunostaining was performed with antibodies against α-actinin for cardiomyocytes (a,b) and PECAM-1 for endothelial cells (c,d), magnification×80 (modified from [57]). (B) Stimulation of cardiomyocyte differentiation of murine E.S. cells by exogenous H_2O_2 (modified from [58]). Incubation of ESCAs generated from ES cells with different concentrations of exogenous H_2O_2 increased cardiomyocyte differentiation as evaluated by counting the number of spontaneously contracting ESCAs. (C) Treatment of ESCAs outgrown on coverslips with the PI 3-K antagonist LY294002 inhibited cardiomyocyte differentiation (b) and decreased intracellular R.O.S. levels (not shown). Upon coadministration of LY294002 with either 10 nM H_2O_2 (c) or 10 μM menadione (which increases intracellular ROS levels) (d) cardiomyocyte differentiation was restored to the control value (a), indicating that the PI-3-kinase is involved in the control of intracellular ROS levels. Pictures in (C) show representative 12-day-old ESCAs stained with an antibody directed against α-actinin. The bar represents 150 μm.

endothelial cells was not affected in the same ESCAs, activation of PI 3-K appears to promote development of cardiomyocytes in a selective manner [57].

Furthermore, PI 3-K has been demonstrated to be involved in the regulation of the neutrophil NAD(P)H oxidase [93]. In ES cell-derived ESCAs a robust endogenous generation of ROS was observed which was due to the expression of a nonphagocytic NAD(P)H oxidase, and was

downregulated during the time course of differentiation [58]. Brief incubation of ESCAs with low concentrations of ROS stimulated cardiomyogenesis [94] (Fig. 5B and C) pointing to a role for ROS as signalling molecules in cardiomyogenesis of ES cells. Preincubation of ESCAs with antagonists of the PI 3-K significantly reduced the intracellular level of ROS, suggesting that PI 3-K may be involved in the regulation of the activity of a non-phagocytic NAD(P)H oxidase in ESCAs, comparable to the situation observed in phagocytic cells. Consequently cardiomyogenesis in ESCAs was restored when PI 3-K inhibitors were coadministered with prooxidants [58].

2.7. NO signalling and cardiogenesis

Nitric oxide (NO) is an ubiquitous signalling molecule, characterised by its high reactivity but self-limiting duration of action. The generation of NO is controlled by NOSs (NOS) of which three isoforms have been identified so far. These enzymes display tissue-specific expression and their activation is also related to the cell status and specific stages during development. We have investigated in detail the expression and cellular role of NO in the heart muscle during embryonic development. Throughout embryonic development of mouse and rat, both isoforms, cytokine-inducible NOS (iNOS) and endothelial NOS (eNOS) were detected in atrial and ventricular cardiomyocytes [95]. Interestingly, a differential expression pattern is observed in atria and ventricles. Both NOS isoforms can be detected at very early stages of embryonic development (starting at E8.5), however, iNOS and eNOS content declined starting with E14.5 in the ventricles, whereas higher levels persist in the atria. At no point during development was expression of neuronal NOS (nNOS) found in the heart muscle. In parallel to the expression pattern of the NOS isoforms, intact NO-dependent signalling cascades in the form of soluble guanylyl cyclase and cGMP were also identified [95]. Because of the expression pattern of NOS and the strong positive staining for NADPH-diaphorase, we next tested whether NO modulated the physiological activity of embryonic cardiomyocytes. In order to generate physiologically intact early-stage embryonic cardiomyocytes and investigate their functional characteristics, the in vitro differentiation system using ES cells was employed [96–98]. With the hanging drop method (Fig. 6) and defined plating periods, early-, but also late-stage embryonic cardiomyocytes can be obtained [96]. The voltage dependent L-type Ca^{2+} current (I_{CaL}) has been used as a sensitive read out to analyse the NO-related modulation with the patch clamp method at the single cell level. The experiments proved that during early stages of development, constitutive iNOS-mediated NO-generation occurred. This led to high levels of intracellular NO and to a fast decrease of the spontaneous beating (negative chronotropy) of cardiomyocytes [99]. Although the physiological relevance of this is not entirely clear, the high intrinsic NO content leads to slowing of the heart rate causing lower consumption of O_2 and substrates. This appears critical for the early embryonic heart, as force of contraction and perfusion with oxygenated blood is still poor at this stage, requiring mechanisms to control the energy consumption. Furthermore, NO was also found to be involved as a signalling molecule in the muscarinic regulation of the heart muscle [100,101] as acetylcholine led to an increase of NO via activation of e-NOS [99]. In parallel to the decline of NOS isoform expression during embryonic development (around E14.5 in ventricle), the constitutive- and hormone-induced production of NO comes to a stop, while the cardiomyocytes switch to a postnatal-like pattern of hormonal regulation [95]. To investigate whether fast cascade signalling pathways might also be involved in the regulation of developmental processes of cardiac cells, the role of NO during early cardiomyogenesis was investigated within murine ESCAs. Incubation of ESCAs with NOS-inhibitors led to a pronounced delay of cardiac differentiation. Thus, besides the above-mentioned important role of NO as a signalling molecule for early heart function, NO-specific action on cardiac differentiation was found. These findings are in agreement with earlier reports, where it was shown that NO in presence of neuronal growth factor (NGF) arrests the proliferative phase and induces neuronal differentiation [102]. The mechanism has been analysed in *Drosophila melanogaster* where NO was shown to have an anti-proliferative effect on embryonic tissues by regulating the critical equilibrium between cell proliferation and differentiation [103].

3. Functional properties and generation of cardiomyocytes from embryonic stem cells

In order to elucidate the cell subtypes as well as time course of cardiac differentiation of ES cells the live colour reporter gene approach has been introduced by our group, and is currently used in a number of laboratories. This approach involves the stable transfection of ES cells with a cardiac-specific promoter/enhancer to drive the expression of the enhanced green fluorescence protein (EGFP) gene. In recent years our group has focused on analysing the expression of ion channels during cardiac development and relating their individual expression pattern with the function of cardiomyocytes [97]. The expression of EGFP under control of cardiac-specific promoters such as cardiac α-actin in combination with the ES cell system has allowed us to characterise the functional characteristics of cardiac precursor cells (Table 1), that is of cardiomyocytes prior to the initiation of the spontaneous contraction [104–106].

After plating of ESCAs according to our protocol (Fig. 6A) fluorescent green areas were first detected about 12 h later. Spontaneously beating clusters coincided with the

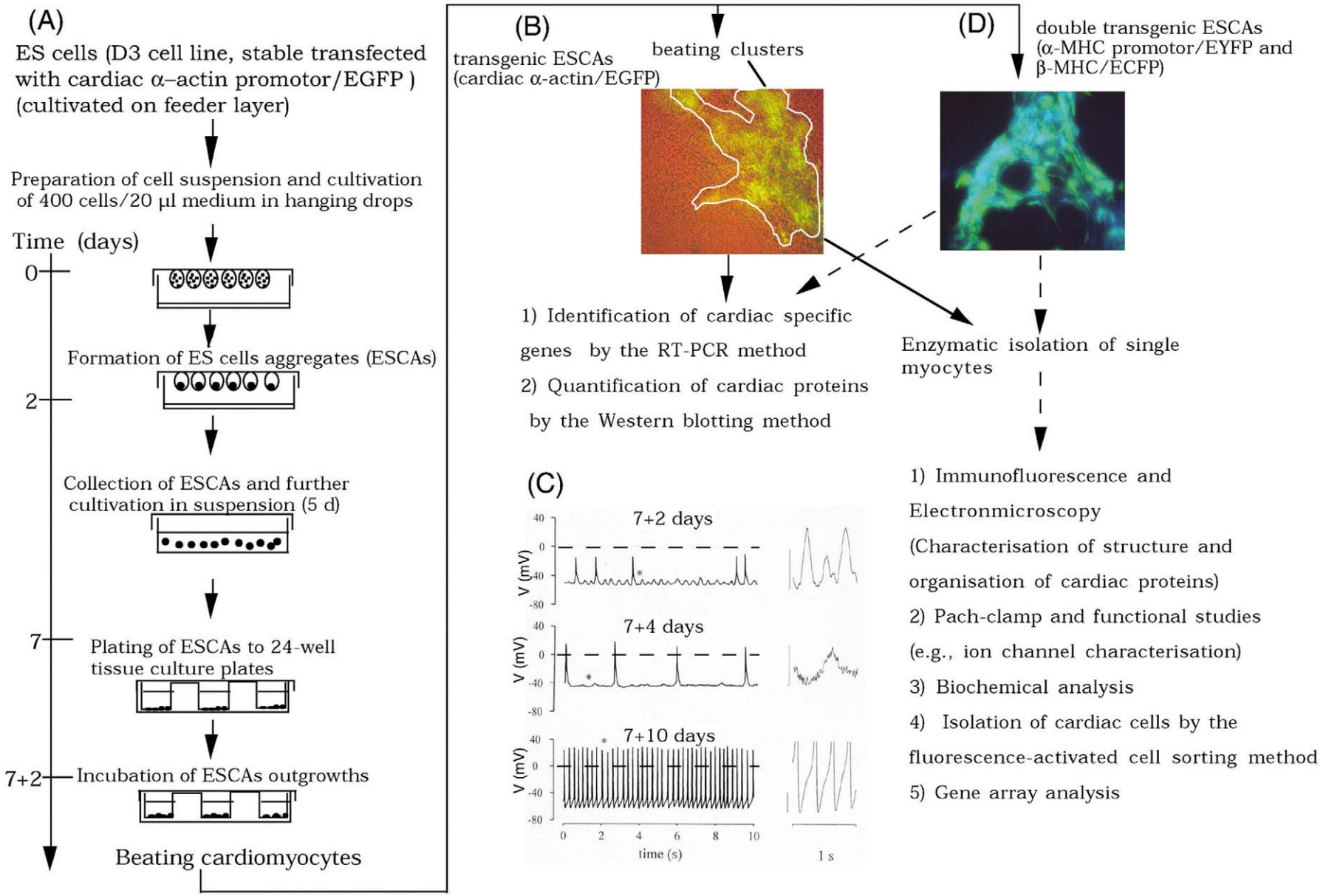

Fig. 6. Protocol for the in vitro differentiation of murine ES cells to cardiomyocytes (A). (B) ES cells were stably transfected with a vector containing GFP under control of the cardiac α-actin promoter. The arrow indicates a typical green beating cardiac cell cluster expressing cardiac specific α-actin. (C) Electrical activity records in spontaneously beating ES cell-derived cardiomyocytes. Upper record: typical recording of early cardiomyocytes from day 7+2 with low resting membrane potentials and action potentials (APs) of small amplitude. Membrane potential fluctations are marked by an asterisk and are shown at extended scales (vertical calibration bar represents 10 mV) on the right were intercalated between APs. Middle record: Cardiomyocytes from day 7+4 displayed typically depolarised resting potentials but larger APs, compared to that of day 7+2. Lower record: Terminal differentiated cardiomyocytes from day 7+10 are pacemaker-like cells displaying an unstable resting potential and the typical diastolic depolarisation (C: modified from [107]). (D) ES cells were co-transfected with two vectors: α-MHC–EYFP and β-MHC–ECFP and selected with G418 in order to obtain stable clones. Differentiation patterns (beating areas) of one of the clones (no. 6) is shown. EYFP and ECFP were detected under a fluorescent microscope with corresponding bandpass filters. The pictures of ESCAs were taken using double snap-shots in order to overlay two colour images. One can see preferentially EYFP or ECFP positive cell clusters as well as cell clusters expressing both (white like), magnification×200.

Table 1
Stage classification and properties of ES cell-derived cardiomyocytes

	Stages				
	0	1	2	3	4
Nomenclature Nomenclature Days of differentiation	Precursor	VEDS 7+1/(2)	EDS Early 7+(2)/3 to 7+4/(5)	EDS Intermediate 7+(5)/6 to 7+8	LDS Late 7+9 to 7+18
Cell types	Uniform	Uniform, only one phenotype		Diversification of different phenotypes	Different phenotypes: ventricular-, atrial-, sinusnodal-, Purkinje-like cells
Currents	I_{Ca} (10–20 pA at 10 mM Ca) Kv channels	I_{Ca}, I_{to}	I_{Ca}, I_{to}, $I_{K,sus}$, $I_f(+/-)$, $I_{K,Ach}$ (slow), I_{KATP}	Specialisation of currents	All currents typical for the different phenotypes
Resting potential (RP)		Depolarised	Relatively depolarised, −50 to −70 mV	Nd	−80 mV (ventricular) about −45 mV (sinusnodal)
Membrane potential (MP) waves	Nd	Yes, +++	+/−	−	−
Action potentials (APs)	No	Small	Larger	Nd	Sinusnodal-like ventricular-like atrial-like Purkinje-like
Overshoot		No	Yes	Nd	Yes
Contractions	−	+	+	+	+++
Forskolin	−	−	+	++	++
Muscarinic stimulation	−	−	+	+	I_{KACh}, anti-β-adrenergic
β-Adrenergic stimulation	−	−	−	+/−	+++
NOS expression			High NOS II, III	Nd	Low NOS III
CGMP content			High cGMP	Nd	Low cGMP

Membrane potential waves are characterised by their small amplitude (<30 mV). The hormonal stimulation was evaluated based on its effect onto the amplitude of peak I_{Ca}; I_{to}, transient outward rectifying K^+ current; $I_{K,sus}$, sustained outward rectifying K^+ current; I_f, hyperpolarisation activated nonselective cation current; $I_{K,ATP}$, ATP-dependent inward rectifying K^+ current; $I_{K,Ach}$, acetylcholine activated inward rectifying K^+ current. I_f: hyperpolarisation-activated nonselective cation current; I_k, ATP-dependent inward rectifying K^+ current; Kv, voltage-gated K^+ channel; $I_{k,Ach}$, acetylcholine-activated inward rectifying K^+ current; nd, not determined.

EGFP positive areas. (Fig. 6B) (for review see [98]). Our electrophysiological experiments clearly showed that at early stages of differentiation (7+1 or 7+2 days, see differentiation protocol, Fig. 6A) the cardiomyocytes were still equipped with a primitive set of ion channels compared to terminally differentiated cardiomyocytes (Fig. 6C, upper record). We therefore speculated as to how the cells initiated spontaneous contractions, known to occur at stage 9.5 days of murine and at 6.5 weeks of human embryonic development. In contrast to the adult heart, early stage murine ES cell-derived cardiomyocytes continued to spontaneously contract even upon complete depolarisation of the membrane potential by perfusion with high K^+ solution [107]. Patch clamp and/or Ca^{2+} measurements demonstrated, that intracellular Ca^{2+} oscillations were responsible for these contractions. These led to small depolarisations of the membrane potential because of activation of a Ca^{2+}-activated nonselective cation current

or the Na^+–Ca^{2+} exchanger (Fig. 6C, upper record) [107]. In contrast, cardiomyocytes from day 7+4 displayed typically depolarised resting potentials but larger action potentials (APs). Since diseased cells presumably recapitulate early embryonic pathways, future studies are aimed at clarifying whether ventricular arrhythmias may be induced by the onset of spontaneous intracellular Ca^{2+} oscillations [108]. Table 1 summarises stage classification and properties of the ES-derived cardiomyocytes. Morphological characterisation of the terminally differentiated ES-derived cardiomyocytes (7+9 to 7+15 days) based on the myofibrillar organisation as well as electrophysiological studies showed a heterogeneous population of cardiomyocytes [98] as pacemaker-like, Purkinje-like, atrial-like and ventricular-like cells were found in the cardiac cell population (for review see [98]). Similar to adult cardiomyocytes, ES cell-derived cardiomyocytes developmentally express cardiac specific genes including α- and β-MHC, cardiac troponin C and atrial natriuretic peptide. Moreover, the electrophysiological characteristics and ion channel expression of ES cell-derived cardiomyocytes is reminiscent of embryonic and postnatal murine cardiomyocytes [96–98,109,110]. In summary the ES-derived cardiomyocytes functionally expressed the L-type Ca^{2+} current, the TTX-sensitive Na^+ current, outwardly rectifying K^+ currents (I_K) and the hyperpolarisation activated nonselective cation (I_f) current [98,104]. The L-type Ca^{2+} current was found to be regulated by both β-adrenergic and muscarinic signalling pathways via cAMP suggesting that intracellular signalling cascades are intact [97,98].

Moreover, EGFP transgenic cardiac cells can be purified by the fluorescence-activated cell sorting method, a well-established method for purification of the CD34+ hematopoietic stem cells for transplantation in cancer disease. In this context, to isolate the subpopulation of ventricular-like cardiomyocytes, ES cells were stably transfected with EGFP under transcriptional control of the ventricular-specific myosin light chain-2v (MLC-2v) promoter and the enhancer element of the cytomegalovirus (CMV(enh)) [111]. Four weeks after initiation of differentiation 25% of the cardiomyocyte population displayed fluorescence. Immunohistochemistry revealed the exclusive cardiomyogenic nature of EGFP-positive cells. Electrophysiological studies showed that preferentially ventricular phenotypes, but neither pacemaker-like nor atrial-like cardiomyocytes, were detected among the EGFP-positive population. After enzymatic digestion of ESCAs and percoll gradient centrifugation the purity was examined by fluorescence-activated cell sorting and showed a 97% pure population of cardiomyocytes [111]. More recently, even more sophisticated approaches involve the usage of regulatory elements or promoters for two different cardiac specific and developmentally dependent genes. Thus, ES cell clones possessing two vectors with EGFP derivatives, enhanced yellow (EYFP) and enhanced cyan fluorescent proteins (ECFP) under the control of the promoters of α- and β-MHC, respectively were employed in our laboratory (Fig. 6D). Expression of these closely related sarcomeric contractile proteins is highly development-dependent and shows patterns of heart chamber specificity during embryonic cardiomyogenesis [112]. The usage of different combinations of cardiac chamber-specific promoters in such a two-marker system could allow for a more precise identification and functional study during ES cell-derived cardiomyogenesis. Finally, in order to generate a sufficient numbers of early cardiac cells for cell therapy of cardiovascular diseases we established a method for the generation of large amounts of ESCAs using the spinner flask culture technique [113] and isolated nearly 1000 ESCAs per spinner flask [113].

Another strategy for generation of cardiac cells from ES cells implicates the use of a cardiac-specific promoter to drive a selection marker i.e. an antibiotic resistance gene [114]. For this purpose a fusion gene consisting of the α-MHC promoter and a cDNA encoding aminoglycoside phosphotransferase was stably transfected into pluripotent ES cells. For cardiac cell selection the stably transfected ESCAs were subjected to geneticin. Immunocytological and ultrastructural analyses demonstrated that the selected cardiomyocytes cultures possess a purity of 99%. Furthermore, transplantation experiments showed that the geneticin selected cardiomyocytes possess the ability to form grafts in the hearts of adult dystrophic mice [114].

4. Generation of cardiomyocytes by growth factors under selective culture conditions

The identification of soluble growth factors, transcription factors and signalling cascades capable of priming selective differentiation of ES cell to cardiac cells is a crucial issue for our understanding of cardiogenesis as well as for the development of stem cell-based therapy of cardiovascular diseases. However, up to now, specific growth factors in combination with culture conditions are not yet available to establish pure cardiomyocyte lineages derived from ES cells. ESCAs represent an optimal model for identification of growth factors promoting selective differentiation. However, since foetal calf serum (FCS) contains several growth factors promoting and inhibiting cardiac development, a defined low-serum medium is essential for identification of growth factors influencing generation of cardiomyocytes. Therefore, we are now trying to establish new serum-free media containing supplementary nonprotein compounds in which ESCAs can survive. In these media the influence of different growth factors alone or in combination on cardiac differentiation of ES cells can be examined. Furthermore, elucidation of important cardiac differentiation signalling pathways of growth factors/receptors promoting the generation of cardiac cells is possible. In this context, preliminary experiments demonstrated that stimulation of ESCAs

cultivated in serum-free media with platelet-derived growth factor (PDGF) significantly promote the generation of cardiomyocytes.

5. Strategies for prevention of immunological rejection of transplanted cells

Although we believe that the combination of genetically manipulative methods with selective culture conditions represent an optimal strategy for establishment of a pure cardiac cell lineage for cell transplantation therapy, one of the concerns for transplantation in humans remains the immunological rejection of the engrafted cardiomyocytes. Although immunosuppression agents, currently used after organ transplantation, can prevent rejection, they are associated with several complications including wound healing, opportunistic infections, drug-related toxicities, skin malignancies, and low-grade lymphomas called post-transplant lymphoproliferative disorders (for review see [6]). Up to now, two strategies to avoid immunosuppressive treatment and its negative side effects are discussed. (i) Reduction or elimination of immunomediated rejection of human ES cells by genetic manipulation. Recently, this strategy has been evaluated demonstrating that the knock-out of the major histocompatibility complex (MHC) class I and class II molecules in mouse ES cells is possible by homologous recombination [115]. However, since MHC class I- and class II-deficient skin grafts were still rejected, it might be necessary to knock-in the MHC genes from the recipient [6]. (ii) Another more theoretical alternative to prevent rejection could be nuclear transfer technology (also known as therapeutic cloning technology). Briefly, a nucleus from a somatic cell from a recipient can be reprogrammed after transfer to an enucleated oocyte. An embryo generated by this method could then be used to establish an ES cell line that express all histocompatibility antigens identical to those of the individual from whom the somatic cell was obtained [6]. However, this technique is not yet appropriately developed and it may also bear unpredictable risks. Moreover, besides the great number of unresolved scientific issues, this approach also remains ethically strongly debated. Future strategies should also clarify the potential of so-called adult stem cells to transdifferentiate under appropriate conditions into other tissue types. Moreover, investigations have also started to identify critical signalling components in order to be able to dedifferentiate adult cells to a pluri-/multipotent state. Finally, immunological rejection of the transplanted cells could be avoided by establishment of embryonic stem cell banks with different human ES cell lines which all differ in their genetic background and histocompatibility.

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