Stimulation of ES-cell-derived cardiomyogenesis and neonatal cardiac cell proliferation by reactive oxygen species and NADPH oxidase

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Summary

After birth the proliferation of cardiac cells declines, and further growth of the heart occurs by hypertrophic cell growth. In the present study the cell proliferation capacity of mouse embryonic stem (ES) cells versus neonatal cardiomyocytes and the effects of reactive oxygen species (ROS) on cardiomyogenesis and cardiac cell proliferation of ES cells was investigated. Low levels of hydrogen peroxide stimulated cardiomyogenesis of ES cells and induced proliferation of cardiomyocytes derived from ES cells and neonatal mice, as investigated by nuclear translocation of cyclin D1, downregulation of $p27^{Kip1}$, phosphorylation of retinoblastoma (Rb), increase of Ki-67 expression and incorporation of BrdU. The observed effects were blunted by the free radical scavengers vitamin E and 2-mercaptoglycin (NMPG). In ES cells ROS induced expression of the cardiac-specific genes encoding α-actin, β-MHC, MLC2a, MLC2v and ANP as well as the transcription factors GATA-4, Nkx-2.5, MEF2C, DTEF-1 and the growth factor BMP-10. During differentiation ES cells expressed the NADPH oxidase isoforms *Nox-1*, *Nox-2* and *Nox-4*. Treatment of cardiac cells with ROS increased Nox-1, Nox-4, p22-phox, p47-phox and p67-phox proteins as well as *Nox-1* and *Nox-4* mRNA, indicating feed-forward regulation of ROS generation. Inhibition of NADPH oxidase with diphenylen iodonium chloride (DPI) and apocynin abolished ROS-induced cardiomyogenesis of ES cells. Our data suggest that proliferation of neonatal and ES-cell-derived cardiac cells involves ROS-mediated signalling cascades and point towards an involvement of NADPH oxidase in cardiovascular differentiation of ES cells.

Key words: Embryonic stem cell, Cardiac cell proliferation, Reactive oxygen species, NADPH oxidase

Introduction

Recently, embryonic stem (ES) cells differentiated in vitro have been suggested as a novel source of cells for transplantation (Dowell et al., 2003; Hassink et al., 2003; Nir et al., 2003). In animal studies ES-cell-derived cardiomyocytes have been shown to form stable intracardiac grafts (Klug et al., 1996), and have been successfully used to improve heart function after cardiac infarction (Behfar et al., 2002). Cardiomyocytes derived from pluripotent ES cells differentiate in successive maturation steps (Sachinidis et al., 2003; Hescheler et al., 1999; Hescheler et al., 2002; Boheler et al., 2002; Wobus et al., 2002; Wobus et al., 1995) and have been discussed to exert considerable proliferative activity for several days after isolation from multicellular embryoid bodies, thus making them a useful tool to investigate the molecular mechanisms of cardiac cell proliferation control as well as gain of cardiomyocyte function (Kehat and Gepstein, 2003). Furthermore, increased knowledge of ES-cell-derived cardiomyocyte proliferation will enable the elaboration of cell culture protocols to generate mass cultures of cardiomyocytes, which may be used in cell replacement therapy.

Currently, not much is known about the signalling mechanisms that initiate differentiation of ES cells towards the cardiomyogenic cell lineage. Furthermore, the regulation of the cell cycle of ES-cell-derived cardiac cells has not been investigated, nor have sufficient analyses been undertaken that compare the proliferation of ES-cell-derived cardiac cells with cells of a commensurable differentiation status, i.e. neonatal cardiomyocytes. The established theory of myocardial development holds that after a limited number of cell divisions, the cardiomyocytes of the developing heart are irreversibly withdrawn from the generation cycle (von Harsdorf, 2001). However, it has been recently shown that cardiomyocytes may regain proliferation capacity after cardiac infarction (Beltrami et al., 2001). Furthermore, recent studies have demonstrated that the adult heart contains a tiny population of cardiomyocytes that retain their proliferative potency, are multipotent and support myocardial regeneration (Beltrami et al., 2003). Cardiac progenitor cells express stem cell antigen-1 but express neither cardiac structural genes nor *Nkx2.5*, and differentiate in vitro in response to the DNA-demethylating agent 5′-azacytidine (Oh et al., 2003). During aortic stenosis in humans, which causes cardiac hypertrophy, growth and differentiation of cardiac stem cells was markedly enhanced, suggesting that cardiac stem cells amplify and commit to the myocyte lineage in response to increased workload (Urbanek et al., 2003). This observation is consistent with the notion that myocyte hyperplasia significantly contributes to cardiac

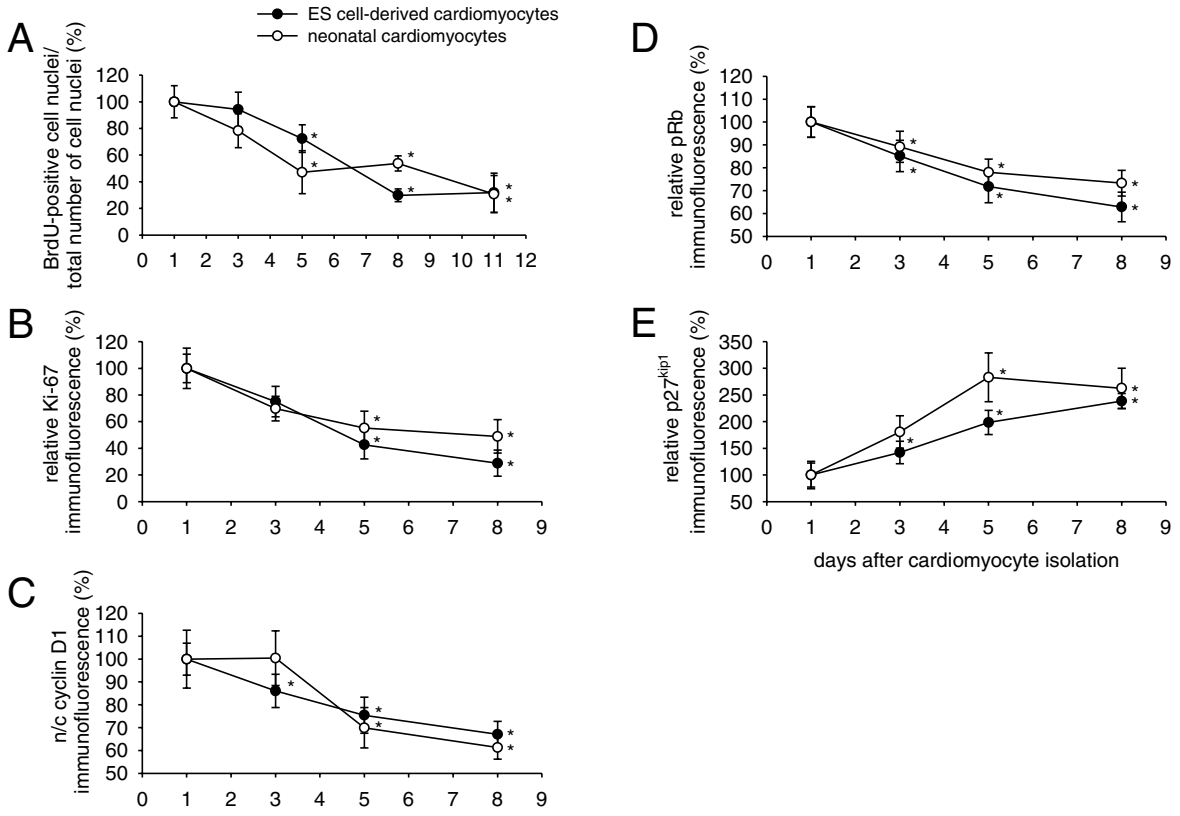

Fig. 1. Decay of the proliferation capacity of ES cell-derived- and neonatal cardiac cells after cell isolation from embryoid bodies and neonatal mouse hearts. (A,B) Decay of the numbers of BrdU-positive cell nuclei of cardiac cells. (C,D) Decay in Ki-67 immunoreactivity in cardiac cells. (E,F) Nuclear/cytoplasmic (n/c) cyclin D1 distribution in cardiac cells. (G,H) Decay in retinoblastoma (Rb) phosphorylation in cardiac cells during different times of cell culture. (I,J) Increase in $p27^{kip1}$ expression in the cell nuclei of cardiac cells during different times of cell culture. *P<0.05, significantly different to day 1 of cell culture.

hypertrophy and accounts for the subpopulation of cycling myocytes (Urbanek et al., 2003). Since ES cells and intrinsic cardiac cells might originate from a common precursor, comparable signalling mechanisms might be used to initiate cardiomyogenic differentiation of both cell types.

We have previously shown that differentiation of ES cells towards the cardiomyogenic cell line is enhanced by ROS generated predominantly by NADPH oxidase. Conversely, cardiomyogenesis in ES cells is inhibited in the presence of free radical scavengers (Sauer et al., 1999). In the present study we investigated whether ROS stimulated the differentiation as well as proliferation of ES-cell-derived cardiomyocytes in comparison to murine neonatal cardiomyocytes. It is shown that ROS enhance differentiation of ES cells towards cardiac cells. The proliferation capacity of ES-cell-derived cardiac cells as well as neonatal cardiomyocytes declines shortly after cell isolation and can be restored through activation of NADPH oxidase by low levels of hydrogen peroxide.

Results

Proliferation capacity of ES-cell-derived cardiac cells versus cardiac cells from neonatal mouse hearts

ES-cell-derived cardiomyocytes can be used for cell replacement therapies of cardiac infarction. Although it is well accepted that cardiac cells lose their proliferation capacity shortly after birth, not much information is available on the proliferation capacity of ES-cell-derived cardiomyocytes compared with cardiac cells isolated from intact neonatal mouse hearts. We therefore assessed the proliferation capacity of ES-cell-derived cardiomyocytes versus neonatal cardiomyocytes and applied several markers for cell proliferation, i.e. BrdU labelling of DNA synthesis, staining of the proliferation markers Ki-67 and cyclin D1, and phosphorylation of retinoblastoma (Rb) protein. In parallel, the increase in the expression of the cyclin-dependent kinase (CDK) inhibitor and marker of cell-cycle arrest $p27^{kip1}$ was determined after cardiac cell isolation. It was apparent that the number of BrdU-positive cell nuclei declined with comparable kinetics within 10 days after cell isolation in ES cell-derived cardiac cells as well as neonatal cardiac cells, indicating a rapid decrease in DNA synthesis within few days (Fig. 1A,B; n=3). Consequently, Ki-67 (Fig. 1B; n=3), nuclear/cytoplasmic distribution of cyclin D1 (Fig. 1C; n=4) as well as phospho-Rb immunoreactivity (Fig. 1D; n=3) declined within days after cell isolation in ES-cell-derived cardiomyocytes and in neonatal cardiac cells. By contrast, upregulation of $p27^{kip1}$ expression (Fig. 1E; n=3) was observed within days in both cardiac cell preparations. In summary, these data demonstrate that the proliferation capacity of ES-cell-derived cardiac cells

is comparable to neonatal cardiac cells and declines within approximately one week after cell isolation.

Stimulation of cardiomyogenesis by H_2O_2

We have previously demonstrated that cardiomyogenesis of ES cells can be stimulated by very low concentrations of ROS (Sauer et al., 1999). To corroborate our previous results, ES-cell-derived embryoid bodies were incubated on day 4 of differentiation for 24 hours with 500 nM H_2O_2 and the number of spontaneously contracting embryoid bodies was counted after plating on day 8 of cell culture (Fig. 2A). It was apparent that treatment with H_2O_2 significantly increased the number of beating embryoid bodies by approximately 50% (n=5), which could be totally abolished in the presence of either the free radical scavenger vitamin E (100 μM) (n=3) or NMPG (100 μM) (n=3). Furthermore treatment with 500 nM H_2O_2 significantly increased protein expression of α-actinin (n=3), MLC2a (n=3) and cMLC1 (n=3) (see Fig. 2C) as well as mRNA expression of cardiac α-actin (n=4), β-MHC (n=4), MLC2a (n=4), MLC-2v (n=3) and ANP (n=4), which clearly demonstrates stimulation of cardiomyogenesis of ES cells by low concentrations of exogenous added ROS (see Fig. 2B).

Increase in NADPH oxidase expression upon treatment of ES-cell-derived cardiomyocytes with H_2O_2

In embryoid bodies, spontaneous generation of ROS occurs mainly through the activity of NADPH oxidase (Sauer et al., 1999). In the present study a transient expression of *Nox1*, *Nox2* and *Nox4* mRNA was demonstrated during the differentiation of embryoid bodies. Maximum expression of *Nox1* was found between day 6 and 8 of differentiation, i.e. during the time when cardiomyogenesis occurs (Fig. 3A; n=3). By contrast, *Nox2* expression peaked at day 12 of differentiation. *Nox4* mRNA was continuously expressed with a maximum around day 14 of cell culture.

Treatment of cardiac cells isolated from embryoid bodies (Fig. 3B) as well as cardiac cells from neonatal mouse hearts (Fig. 3C) with 100 nM H_2O_2 resulted in significant upregulation of the NADPH oxidase subunits Nox-1 (n=5), Nox-4 (n=4), p22-phox (n=4), p47-phox (n=4) and p67-phox (n=4). Correspondingly, treatment of embryoid bodies with 500 nM H_2O_2 resulted in increased *Nox1* and *Nox4* mRNA expression (see Fig. 3D) and an increased ROS generation 24 hours thereafter, which amounted to 152±21% of the untreated control (n=5). To investigate the impact of NADPH oxidase on ES-cell-derived cardiomyogenesis, embryoid bodies were treated from day 4 to day 8 of cell culture in the presence of the NADPH oxidase inhibitors DPI (100 nM) and apocyin (10 μM) (see Fig. 3E; n=4). This treatment totally blunted the stimulation of cardiomyogenesis observed with 500 nM H_2O_2 and depressed cardiomyogenesis in the control, although this effect did not reach statistical significance.

Redox regulation of cardiac-specific transcription factors and BMP-10

The transcription factors MEF 2C, DTEF, GATA-4 and Nkx-2.5 as well as the growth factor BMP-10 have been shown to be involved in embryonic heart development and cardiomyogenesis of ES cells. Since the data of our study indicated that cardiomyogenesis of ES cells is regulated by the intracellular redox state, we investigated the ROS-induced

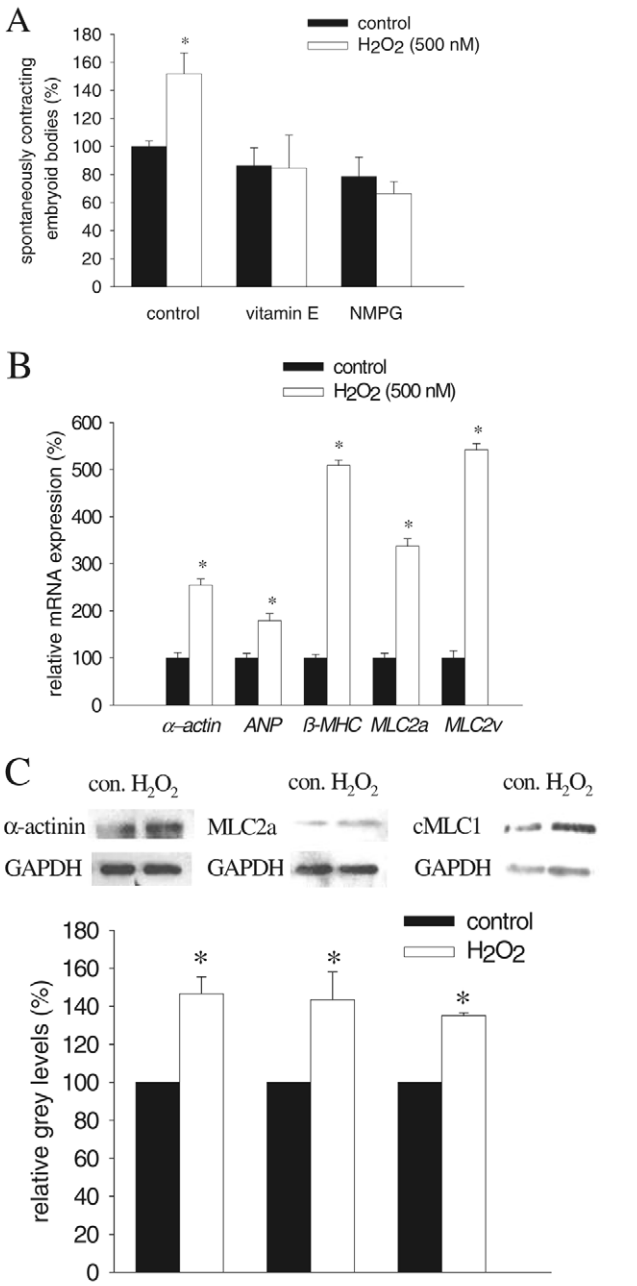

Fig. 2. Stimulation of cardiomyogenesis of ES cells by H_2O_2. (A) ES-cell-derived embryoid bodies were treated on day 4 of differentiation with 500 nM H_2O_2 either in the presence and absence of the antioxidants vitamin E (100 μM) and NMPG (100 μM). H_2O_2 significantly increased the number of spontaneously contracting embryoid bodies on day 8 of cell culture, which was totally abolished in the presence of antioxidants. (B) Treatment of embryoid bodies with H_2O_2 significantly upregulated the cardiac-specific genes α-actin, *ANP*, *β-MHC*, *MLC2a* and *MLC2v*. (C) Upregulation of α-actinin, MLC2a and cMLC1 protein by H_2O_2. The bar chart shows densitometric analyses of three independent experiments. *P<0.05, significantly different to the untreated control.

protein expression of MEF2C, Nkx-2.5 and GATA-4 (Fig. 4). Furthermore mRNA expression of *Mef2c*, *Nkx2.5*, *Gata4*, *DTEF* and *Bmp10* in the absence versus presence of free

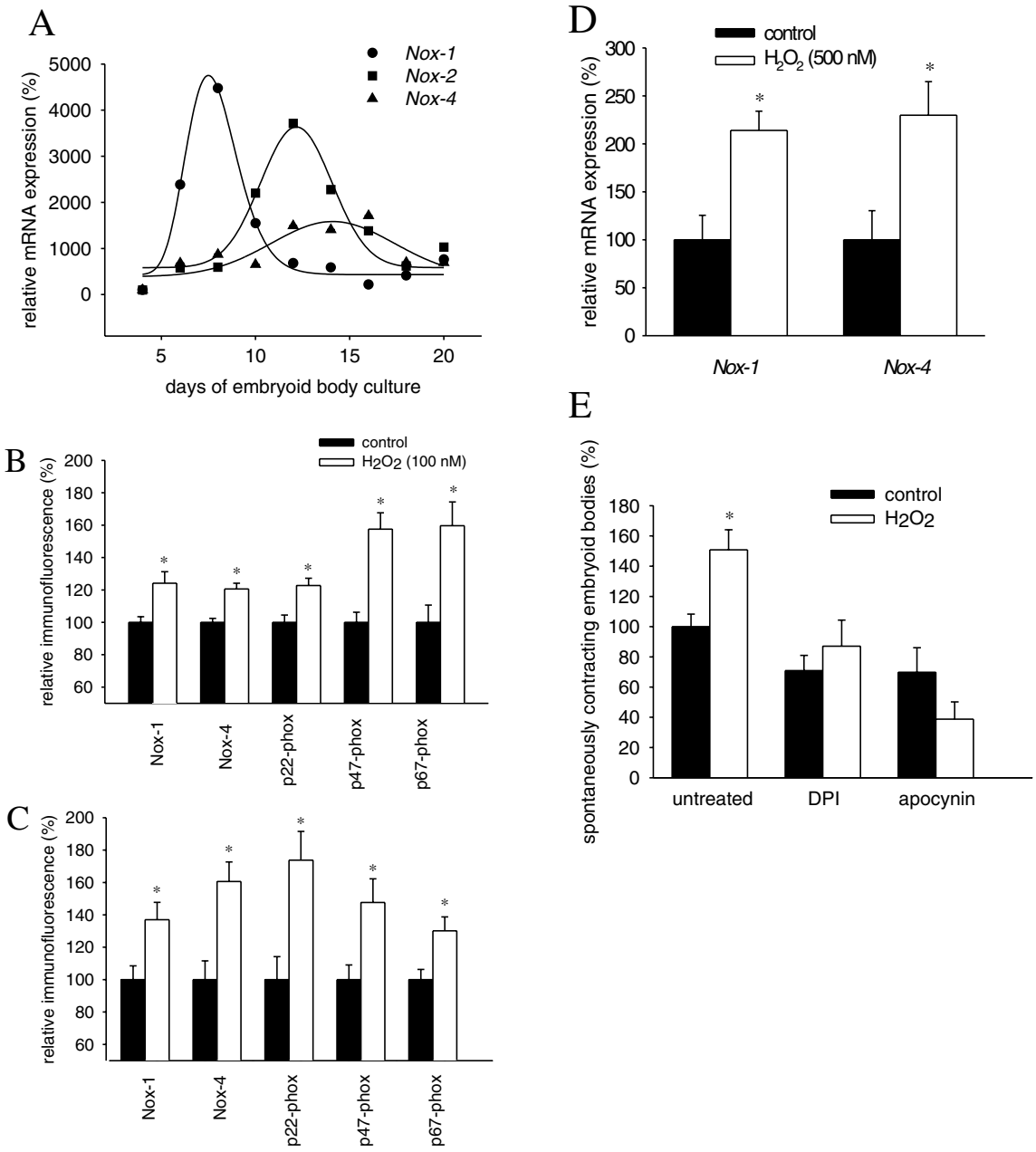

Fig. 3. Role of the NADPH oxidase in cardiomyogenesis of ES cells. (A) mRNA expression of Nox isoforms during ES-cell differentiation within embryoid bodies. Effects of H_2O_2 on protein expression of NADPH oxidase subunits in ES cell-derived cardiac cells (B) and neonatal cardiac cells isolated from mouse hearts (C). (D) Effects of H_2O_2 on *Nox-1* and *Nox-4* mRNA expression in embryoid bodies. (E) Inhibition of H_2O_2-induced cardiomyogenesis by the NADPH oxidase antagonists DPI (100 nM) and apocynin (10 μM). *P<0.05, significantly different to the untreated control.

radical scavengers was assessed (Fig. 5A-E). Treatment of embryoid bodies on day 4 of differentiation with 500 nM H_2O_2 resulted in increased protein expression of MEF 2C (n=3), Nkx-2.5 (n=3) and GATA-4 (n=3) as well as mRNA upregulation of *Mef2c* (n=6) and *DTEF* (n=4) (see Fig. 5A,B), *Gata4* (n=4) and *Nkx2.5* (n=4) (see Fig. 5C,D) as well as *Bmp10* (n=4) (see Fig. 5E) mRNA. The effect of H_2O_2 on mRNA expression of *Mef2c* and *DTEF* was significantly inhibited in the presence of the free radical scavengers vitamin E (100 μM) and NMPG (100 μM). Interestingly, treatment of embryoid bodies with vitamin E and NMPG significantly upregulated the expression of *Gata4*, *Nkx2.5* and *Bmp10* above levels observed in the untreated control as well as that in H_2O_2-treated samples, indicating regulation by redox change rather than by absolute ROS concentration.

Induction of cardiac cell proliferation by ROS

To further validate the stimulation of cardiomyocyte proliferation by ROS, cardiomyocytes were isolated from embryoid bodies as well as neonatal mouse hearts, plated at a

density of 3 cardiomyocytes/mm^2 onto coverslips and treated on day 4 after isolation for 24 hours with 100 nM H_2O_2. After 24 hours, the number of cardiomyocytes was counted by microscopic inspection (Fig. 6A,B). It was found that treatment with H_2O_2 increased the number of ES-cell-derived cardiomyocytes and neonatal cardiomyocytes by 55±15% and 30±9%, respectively, which clearly indicates that low levels of ROS stimulate cell proliferation of ES-cell-derived as well as neonatal cardiomyocytes. To corroborate these findings, FACS analysis was performed with H_2O_2-treated and untreated embryoid bodies after enzymatic dissociation and staining of cardiac cells with α-actinin on day 8 of differentiation. In the H_2O_2-treated samples 5.3±1.1% cardiac cells per embryoid body were observed compared with 3.1±0.5% in the untreated control, representing an increase in cardiac cell numbers of ~70% (n=6). Comparable numbers of cardiac cells in embryoid bodies have previously been reported by other authors (Buesen et al., 2004; Muller et al., 2000). Stimulation of cell proliferation by ROS should be associated with increased expression of proliferation markers as well as decreased expression of the CDK inhibitor p27^{kip1}. It was shown that BrdU incorporation (Fig. 7A,B; n=4), nuclear translocation of Cyclin D1 (Fig. 7C,D; n=5), expression of Ki-67 (Fig. 7E,F; n=4) and phosphorylation of Rb (Fig. 7G,H; n=5) was significantly increased upon treatment with 100 nM H_2O_2 both in neonatal and ES-cell-derived cardiac cells. By contrast, decreased expression of p27^{kip1} (Fig. 7I,J; n=6) was observed, indicating relief from cell-cycle arrest. Hence our data clearly demonstrate that proliferation of neonatal as well as ES-cell-derived cardiac cells can be stimulated by low concentrations of ROS.

Discussion

Cardiomyoplasty will achieve dissatisfactory results until we have gained sufficient knowledge of the physiological parameters that control cardiac cell survival and proliferation at the site of cell transplantation. Although ES cells are advertised as a promising source of cardiac cells for cell transplantation in numerous articles, almost nothing is known about their proliferation capacity in relation to cells of comparable differentiation state, i.e. embryonic or neonatal cardiomyocytes, nor is there any information available about mechanisms of proliferation control. There is general consent that cardiac cells lose their capacity to divide shortly after birth for unknown reasons. Whether a similar decline in the proliferation capacity of ES-cell-derived cardiomyocytes occurs has not yet been thoroughly investigated. To fill this gap we have assessed the proliferation capacity of mouse ES-cell-derived cardiomyocytes versus neonatal cells by clonogenicity assays as well as determination of proliferation markers (BrdU, cyclin D1, Ki-67, pRb) and p27^{kip1} as a marker of cell-cycle arrest. Our data clearly demonstrate that ES-cell-derived cardiomyocytes follow a comparable time course of cell-cycle arrest as observed in neonatal cells which is in good concordance with data observed from intact hearts (Pasumarthi and Field, 2002). It also demonstrates that significant proliferation of ES cells cannot be expected in approaches of cardiomyoplasty after cardiac infarction, thus requiring large numbers of predifferentiated cardiac cells for cell transplantation approaches.

Having investigated the time course of the decline in proliferation capacity of cardiac cells derived from ES cells as well as mouse neonatal cardiomyocytes we assessed possible mechanisms of cell-cycle reactivation, which should give clues to the understanding of the mechanisms of cardiomyogenesis of ES cells as well as the control of embryonic cardiomyocyte differentiation and proliferation. Previously we have shown that the capacitiy of ES cells to differentiate into the cardiomyogenic cell lineage was critically dependent on the redox state of differentiating ES cells, i.e. cardiomyogenesis could be induced with low levels of exogenously added ROS (Sauer et al., 1999). These data were corroborated by a recent elegant study which demonstrated that the NADPH oxidase Nox-4 drives cardiac differentiation of ES cells (Li et al., 2006). Indeed, the data of the present study showed that nanomolar concentrations of ROS not only stimulated cardiomyogenesis in ES cells, but also induced cell-cycle reentry of ES-cell-derived and neonatal cardiomyocytes, thus clearly indicating that cell-cycle regulation by ROS follows

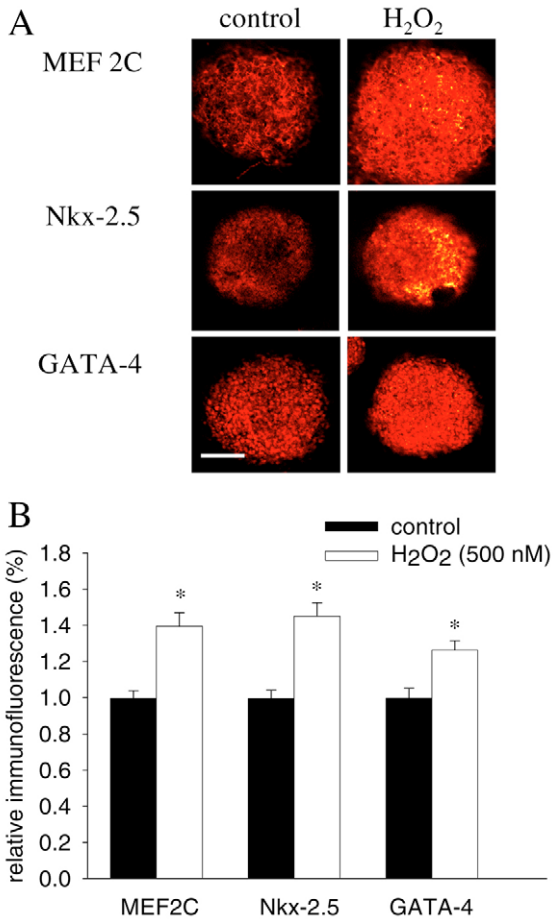

Fig. 4. Semiquantitative analysis of MEF2C, Nkx-2.5 and GATA-4 protein expression 24 hours after treatment with 500 nM H_2O_2. Untreated and treated embryoid bodies were labelled with antibodies directed against the respective protein and immunofluorescence corrected for background fluorescence was quantified by confocal laser scanning microscopy and computer-assisted image analysis. The images show representative immunostainings. Bar, 200 μm. The bar chart represents pooled data from three experiments. *P<0.05, significantly different to the untreated control.

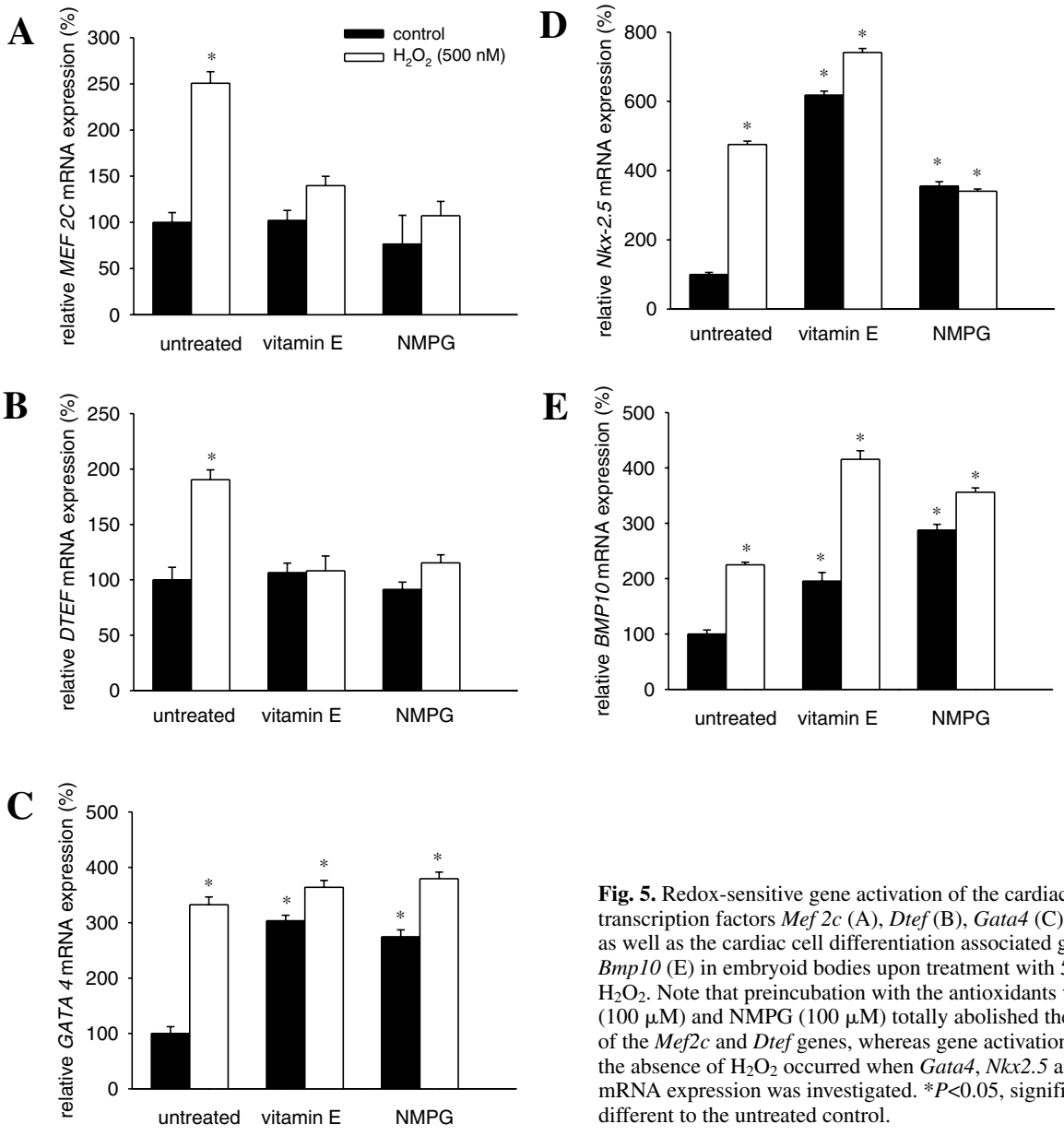

Fig. 5. Redox-sensitive gene activation of the cardiac-specific transcription factors *Mef 2c* (A), *Dtef* (B), *Gata4* (C), *Nkx2.5* (D) as well as the cardiac cell differentiation associated growth factor *Bmp10* (E) in embryoid bodies upon treatment with 500 nM H_2O_2. Note that preincubation with the antioxidants vitamin E (100 μM) and NMPG (100 μM) totally abolished the activation of the *Mef2c* and *Dtef* genes, whereas gene activation already in the absence of H_2O_2 occurred when *Gata4*, *Nkx2.5* and *Bmp10* mRNA expression was investigated. **P*<0.05, significantly different to the untreated control.

comparable mechanisms in both cell systems. Interestingly, treatment of cardiomyocytes from both preparations used in the present study with H_2O_2 significantly increased expression of *Nox1* and *Nox4* mRNA as well as p22-phox, p47-phox and p67-phox protein expression, which indicates a feed-forward cycle of ROS generation that might be required for prolonged cell-cycle activity of cardiac cells. NOX enzymes classically generate $O_2^{\cdot-}$ which is then degraded to H_2O_2 by Cu/Zn superoxide dismutase (SOD) and further to water by catalase. It may be assumed that ES cells tightly regulate their anti-oxidative capacity to avoid oxidative stress without disturbing their capability of reacting to small changes in ROS levels with the activation of distinct signal transduction cascades. Although expression of antioxidant enzymes in mouse ES cells has not yet been investigated, it is very likely that these enzymes are present. In human ES cells SOD and catalase expression has been recently demonstrated. In undifferentiated human ES cells, catalase is highly expressed, whereas in later stages (1-2 weeks) an upregulation of Cu/Zn SOD occurs (Cho et al., 2006). Downregulation of catalase and parallel upregulation of SOD would result in increased H_2O_2 levels inside cells. From this it can be concluded that H_2O_2 is the ROS that regulates cardiomyogenesis in ES cells because the hydroxyl radical, which can be generated from H_2O_2 by the Fenton reaction, is highly toxic and therefore hardly plays a role in signal transduction cascades.

Stimulation of cardiomyogenesis by exogenous ROS should not only increase the number of beating foci of cardiomyocytes but also enhance the expression of cardiac-specific genes and transcription factors. Indeed, treatment of 4-day-old undifferentiated embryoid bodies with H_2O_2 resulted in significant upregulation of expression of the genes encoding MLC2a, MLC2v, α-actin, ANP and β-MHC as well as the cardiac-specific transcription factors MEF2C, DTEF, Nkx-2.5,

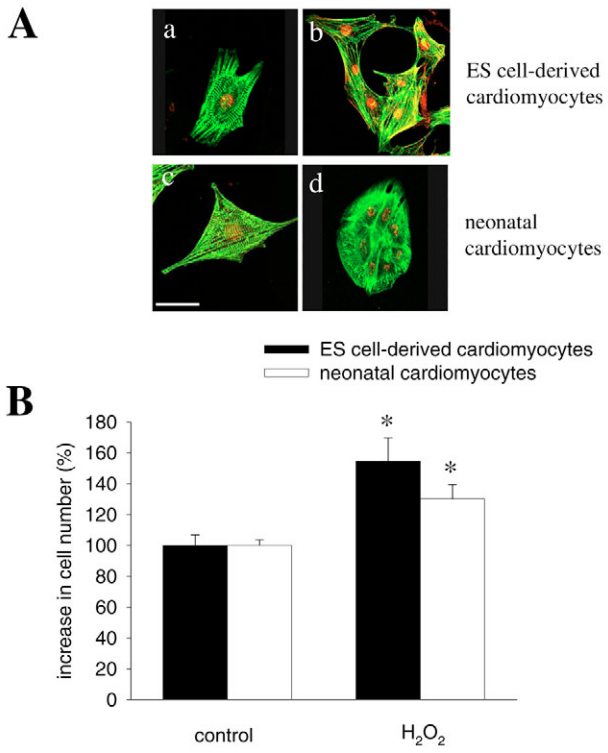

Fig. 6. Increase of ES-cell-derived- and neonatal cardiac cell numbers following treatment with 100 nM H_2O_2. Cardiac cells were treated with H_2O_2 for 24 hours. Subsequently, cell numbers were analyzed following immunohistochemical staining for sarcomeric α-actinin and Sytox Green counterstaining cell nuclei by microscopical inspection. The images in panel A show representative ES-cell-derived cardiomyocytes (a,b) and neonatal cardiomyocytes (c,d), which remained either untreated (a,c) or were treated with H_2O_2 (b,d). Bar, 20 μm. (B) Mean ± s.e.m. of 11 experiments. *P<0.05, significantly different to the untreated control.

GATA-4 and the growth factor BMP-10, which has been previously shown to be critically involved in embryonic heart formation. The increase in *Mef2c* and *DTEF* mRNA expression was significantly inhibited in the presence of free radical scavengers, which indicates that ROS are either directly acting on the level of the transcription factors or are interfering with upstream signalling cascades. Interestingly, preincubation with free radical scavengers increased *Gata4*, *Nkx2.5* and *Bmp10* expression in the absence of H_2O_2 and resulted in an even more pronounced increase of mRNA expression in the H_2O_2-treated samples. This could indicate that these factors are regulated by redox changes rather than absolute increase or decrease of intracellular ROS levels.

Since a certain degree of cardiomyogenesis is also present in the absence of exogenous ROS addition, it is hypothesized that differentiating ES cells are capable of endogenously generating ROS during cardiomyogenesis. In this respect, our data demonstrated that *Nox1*, *Nox2* and *Nox4* are expressed during the time course of differentiation thus corroborating our previous results demonstrating that differentiating ES cells generate significant amounts of ROS (Sauer et al., 1999). Interestingly, *Nox1* expression peaked around day 6-8 of differentiation, i.e. just during the time where cardiomyogenesis occurs, whereas *Nox2* expression had its maximum on day 12; at this time leukocytes are differentiated in embryoid bodies (M. Wartenberg, GKSS Research Center, Teltow, Germany, personal communication). *Nox4* expression displayed a gradual increase in expression around day 14, with a subsequent decline. From these data it can be speculated that *Nox1* is the most important Nox enzyme for cardiomyogenesis. The impact of ROS generated by NADPH oxidase for cardiomyogenesis of ES cells was further underscored by experiments where this enzyme was inhibited by DPI and apocynin. Both agents efficiently inhibited cardiomyocyte differentiation, thus pointing towards a role of NADPH oxidase in cardiomyogenesis of ES cells. Furthermore, cell-cycle stimulation of ES-cell-derived cardiomyocytes as well as neonatal cardiomyocytes was abolished when cells were preincubated with free radical scavengers, which suggests a distinct role of ROS for cardiomyogenesis as well as cardiac cell proliferation control.

In contrast to endothelial and smooth muscle cells, where expression and physiological function of NADPH oxidase has been investigated in detail (Griendling, 2004), the sources of ROS in cardiac cells are not well described nor is there any indication for a role of NADPH oxidase during embryonic heart development. Most recent studies have focussed on the crucial role of oxidative stress in cardiac pathophysiology. In this respect, it has been previously shown that there is increased myocardial NADPH oxidase activity during human heart failure, i.e. the NADPH oxidase subunits p22-phox, Nox-2, p67-phox and p47-phox were all expressed at the mRNA and protein level in cardiomyocytes of both nonfailing and failing hearts (Heymes et al., 2003). NADPH-oxidase-derived ROS were suggested as the causative agents for ventricular dysfunction and subsequent chronic congestive heart failure (Warnholtz and Munzel, 2003), and activation of NADPH oxidase was demonstrated during progression of cardiac hypertrophy to failure (Li et al., 2002). Furthermore, expression of Nox-2 has been demonstrated in human cardiomyocytes and was shown to be upregulated during acute myocardial infarction (Krijnen et al., 2003). Recently, it has been demonstrated that NAD(P)H oxidase 4 mediates transforming growth factor-β1-induced differentiation of cardiac fibroblasts into myofibroblasts, which may represent a major cause of the development of cardiac fibrosis (Cucoranu et al., 2005).

It has been suggested that, during pathologic states of cardiac disease, signalling pathways developed during embryogenesis are reactivated (Kuwahara et al., 2003). In this sense, activation of NADPH oxidase during cardiomyopathy may represent a stimulus for cell-cycle activation that is – as demonstrated by the present study – sufficient to initiate the cell cycle in ES-cell-derived cardiac cells and neonatal cells but not to achieve cell division in hearts of adult individuals. In light of our previous observations (Sauer et al., 1999) and the experiments of the present study, it may be assumed that a finely tuned interplay of oxidant-induced and redox change-mediated cardiac transcription factor activation is required for proper induction of cardiomyogenesis in ES cells. As previously pointed out, comparable alterations in the intracellular redox state of cardiac cells and tissues may occur during cardiac infarction, inflammation, hypertrophy and heart failure (Sauer and Wartenberg, 2005). Hence an alteration of intracellular ROS levels may not only represent the stimulus

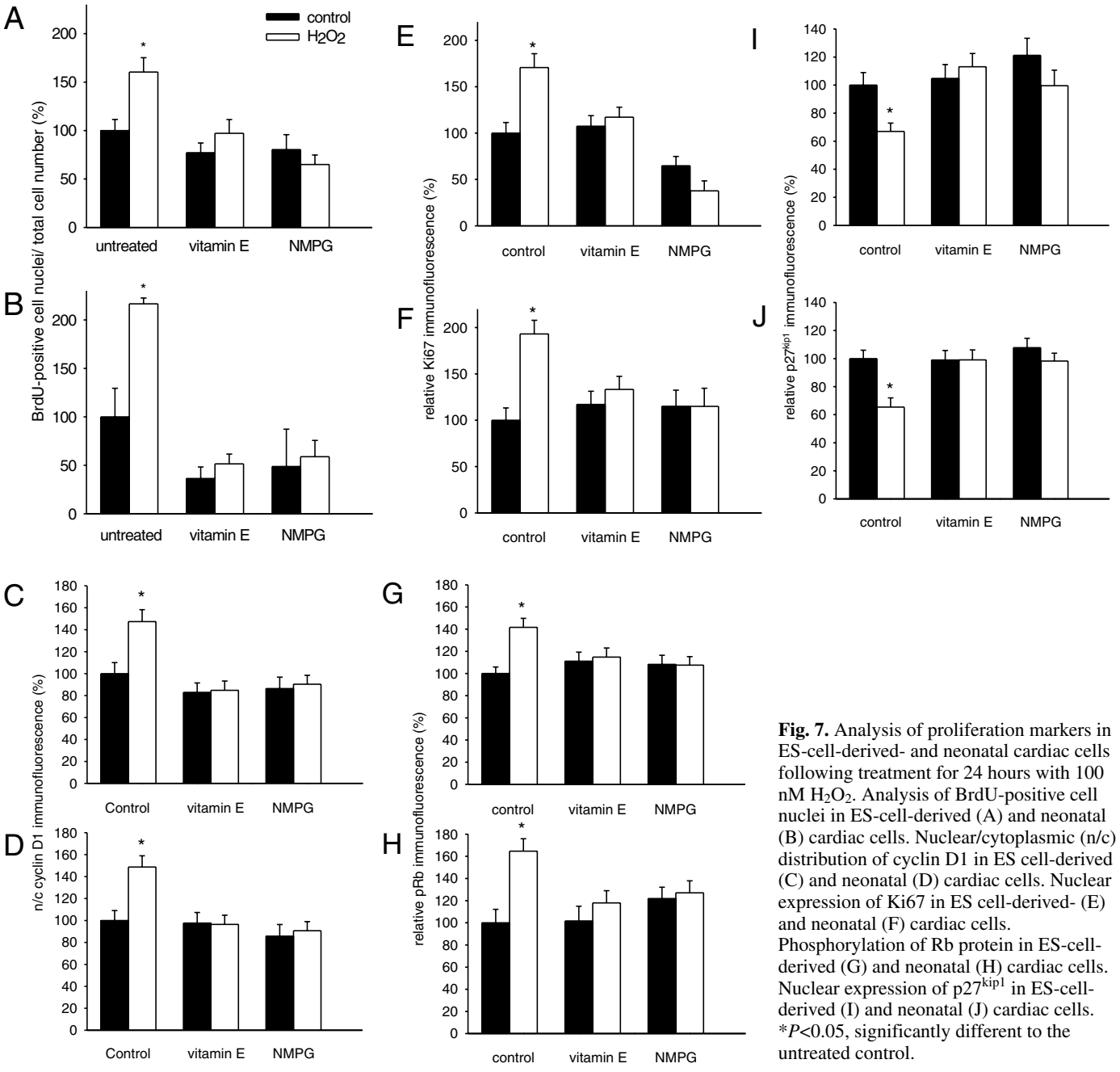

Fig. 7. Analysis of proliferation markers in ES-cell-derived- and neonatal cardiac cells following treatment for 24 hours with 100 nM H_2O_2. Analysis of BrdU-positive cell nuclei in ES-cell-derived (A) and neonatal (B) cardiac cells. Nuclear/cytoplasmic (n/c) distribution of cyclin D1 in ES cell-derived (C) and neonatal (D) cardiac cells. Nuclear expression of Ki67 in ES cell-derived- (E) and neonatal (F) cardiac cells. Phosphorylation of Rb protein in ES-cell-derived (G) and neonatal (H) cardiac cells. Nuclear expression of $p27^{kip1}$ in ES-cell-derived (I) and neonatal (J) cardiac cells. *P<0.05, significantly different to the untreated control.

for cardiomyogenic differentiation of ES cells, but may likewise provide the signal that induces intrinsic cardiac stem cells to divide and differentiate towards mature cardiac cells, thereby supporting their job in cardiac repair and survival.

Materials and Methods

Materials

N-(2-mercapto-propionyl)-glycine (NMPG), diphenylene iodonium chloride (DPI) and apocynin were purchased from Sigma, Deisenhofen, Germany. Trolox (water-soluble vitamin E) was obtained from Calbiochem (Bad Soden, Germany).

Spinner-culture technique for cultivation of embryoid bodies

To obtain embryoid bodies, ES cells (line CCE) were grown on mitotically inactivated feeder layers of primary murine embryonic fibroblasts in Iscove's medium (Gibco, Live Technologies, Helgerman Court, MD) supplemented with 18% heat-inactivated (56°C, 30 minutes) foetal calf serum (FCS) (Sigma), 2 mM glutamine, (PAA, Cölbe, Germany), 100 μM β-mercaptoethanol (Sigma, Deisenhofen, Germany), 1% (v/v) NEA non-essential amino acids stock solution (100×) (Biochrom, Berlin, Germany), 0.8% (v/v) MEM amino acids (50×) (Biochrom), 1 mM Na^+-pyruvate (Biochrom), 0.25% (v/v) penicillin/streptomycin (200×) (Biochrom) and 1000 U/ml LIF (Chemicon, Hampshire, UK) in a humidified environment containing 5% CO_2 at 37°C, and passaged every 2-3 days. At day 0 of differentiation, adherent cells were enzymatically dissociated using 0.05% trypsin-EDTA in phosphate-buffered saline (PBS) (Gibco), and seeded at a density of 3×10^6 cells ml^{-1} in 250 ml siliconized spinner flasks (Integra Biosciences, Fernwald, Germany) containing 100 ml Iscove's medium supplemented with the same additives as described above. Following 24 hours, 150 ml medium was added to give a final volume of 250 ml. The spinner flask medium was stirred at 20 rpm using a stirrer system (Integra Biosciences), and 150 ml cell culture medium was exchanged every day.

Isolation of ES-cell-derived cardiomyocytes from embryoid bodies and neonatal cardiac cells from mouse hearts

Embryoid bodies were removed from spinner flasks and plated onto cell culture

dishes. Cardiomyocytes were isolated from outgrown, spontaneously beating embryoid bodies as previously described (Reinecke et al., 1999). In brief, beating foci were cut out from 8-day-old embryoid bodies using a canula and placed into isolation buffer (119.8 mM NaCl, 20 mM taurine, 10 mM HEPES, 5 mM NaH_2PO_4, 20 mM glucose, 5.4 mM KCl, 5 mM $MgSO_4$, pH 6.9) supplemented with 1 mg/ml collagenase B (Boehringer Ingelheim, Germany). After incubation for 20 minutes at 37°C, single cells were dispersed, centrifuged at 664 ***g*** and resuspended in cell culture medium. To achieve pure cell fractions enriched with cardiomyocytes, cells were placed on top of a Ficoll gradient and centrifuged at 180 ***g*** for 25 minutes. This treatment resulted in a cell pellet of approximately 80% pure cardiac cells. A comparable procedure was applied for the isolation of neonatal cardiac cells. In brief, 3-day-old neonatal mice were killed by decapitation. The atria and great vessels were trimmed and discarded. The ventricles were cut in 2-3 mm^3 pieces and incubated (37°C, 25 minutes) in isolation buffer supplemented with collagenase B. Isolated cells were enriched by Ficoll density gradient centrifugation as described above.

Immunohistochemistry

Immunohistochemistry was performed with cardiomyocytes enzymatically dissociated from either ES cell-derived embryoid bodies or neonatal mouse hearts. Primary antibodies used were as follows: monoclonal mouse anti-sarcomeric α-actinin (dilution 1:200) (Sigma); polyclonal rabbit anti-cyclin D1 (dilution 1:50) (Chemicon); polyclonal rabbit anti-$p27^{Kip1}$ (dilution 1:200) (Chemicon); polyclonal rabbit anti-phospho retinoblastoma (Rb) (dilution 1:200) (Cell Signaling, Frankfurt, Germany); monoclonal rat anti-BrdU (dilution 1:100) (Abcam, Cambridge, UK); polyclonal rabbit anti GATA-4 (dilution 1:100) (Chemicon); polyclonal goat anti-MEF2C and Nkx-2.5 (dilution 1:50) (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal rabbit anti-p47-phox (dilution 1:20) polyclonal goat anti Nox-1, Nox-4, p22-phox and p67-phox antibodies (all from Santa Cruz Biotechnology) (dilution 1:20) were used. Cardiomyocytes on coverslips were fixed in ice-cold methanol for 20 minutes at –20°C, and washed with PBS containing 0.1% Triton X-100 (PBST) (Sigma). Blocking against unspecific binding was performed for 60 minutes with 10% fat-free milk powder dissolved in 0.01% PBST. The tissues were subsequently incubated for 60 minutes (Ki-67 and NADPH oxidase subunits overnight at 6°C) with primary antibodies dissolved in PBS supplemented with 10% milk powder in 0.01% PBST. The cells were thereafter washed three times with PBST (0.01% Triton) and reincubated with either a Cy5-conjugated sheep anti-mouse IgG F(ab')2-fragment, a Cy5-conjugated anti-rat IgG (H+L), a FITC-conjugated goat anti-mouse IgG (Fab-fragment), a Cy5-conjugated mouse anti-goat IgG (H+L) or a Cy3-conjugated donkey anti-goat IgG (all from Dianova, Hamburg, Germany) at a dilution of 1:200 in PBS containing 10% fat-free milk powder in 0.01% PBST. After washing three times in PBST (0.01% Triton) the tissues were stored in PBS until inspection. Fluorescence recordings were performed by means of a confocal laser scanning setup (Leica LSM SP2, AOBS, Bensheim, Germany). The fluorescence values in the respective optical section were evaluated by the image analysis software of the confocal setup.

FACS analysis

Eight-day-old embryoid bodies outgrown on cell culture dishes were enzymatically digested using collagenase B (Boehringer) (1 mg collagenase B/ml buffer) washed once in PBS, centrifuged at 664 ***g*** and fixed in ice-cold methanol. A number of 1×10^6 cells were blocked against unspecific binding in 0.01% PBST supplemented with 10% FCS and stained for 60 minutes with an antibody directed against sarcomeric α-actinin (dilution 1:200) (Sigma). As secondary antibody, a FITC-conjugated anti-mouse IgG antibody (Dianova) was used. FACS analysis was performed using a Becton-Dickinson FACS Calibur Flow Cytometry System (Heidelberg, Germany) and the Cell Quest Pro analysis software (Becton Dickinson).

Western blot analysis

Cells, controls and H_2O_2-stimulated samples, were washed in phosphate-buffered saline (PBS, pH 7.4) once and lysed in ice-cold lysis buffer containing 20 mM Tris-HCL pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 25 μg/ml leupeptin, 1 mM sodium orthovanadate (Sigma) and 1% phosphatase inhibitor cocktail (Sigma). The cell lysates were sonicated twice for 5-10 seconds per cycle, incubated on ice for 10 minutes and centrifuged at 10,000 ***g*** for 15 minutes at 4°C. The supernatant was used for protein determination. Aliquots of 50 μg of protein were separated in 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. The membranes were blocked with 5% dried fat-free milk in Tris-buffered saline containing 0.1% Tween 20. Incubations with primary antibodies were carried out at 4°C overnight using mouse monoclonal antibodies directed against cardiac myosin light chain (cMLC1) (dilution of 1:200) (Abcam), myosin regulatory light chain 2a (MLC2a) (dilution of 1:1000) (Santa Cruz), and α-actinin (dilution of 1:1000) (Abcam). After washing, membranes were incubated with the respective HRP-conjugated secondary antibody for 1 hour at 4°C. Protein expression was detected by ECL system (Amersham Life Science, Munich, Germany) and protein bands visualized by film exposure. GAPDH was used as an internal control.

Quantitative RT-PCR

Total RNA from CCE embryoid bodies treated for 24 hours with the substances indicated was prepared using the Trizol (Invitrogen, Karlsruhe, Germany) method followed by genomic DNA digestion using DNAse I (Invitrogen). Total RNA concentration was determined by the OD_{260nm} method. cDNA synthesis was performed using 2 μg RNA with MMLV RT (Invitrogen). Primer concentration for qPCR was 300 nM. Primer (Invitrogen) sequences were as follows: BMP 10 fwd: 5′-ACC AGA CGT TGG CAA AAG TCA GGC-3′, rev: 5′- GAT GAT CCA GGA GTC CCA CCC AAT-3′; DTEF1 fwd: 5′-CCC GAA CGC TTT CTT CCT TGT C-3′, rev: 5′-ACC TTG GTG GAG ACG CTG ATG-3′; GATA-4 fwd: 5′-TCA AAC CAG AAA ACG GAA GC-3′, rev: 5′-GTG GCA TTG CTG GAG TTA CC-3′; MEF2C fwd: 5′-GTG GCA TTG CTG GAG TTA CC-3′, rev: 5′-TAT TCC TCT GCA GAG ACG GG-3′; Nkx-2.5 fwd: 5′-CCA CTC TCT GCT ACC CAC CT-3′, rev: 5′-CCA GGT TCA GGA TGT CTT TGA-3′; Nox-1 fwd: 5′-AAT GCC CAG GAT CGA GGT-3′, rev: 5′-GAT GGA AGC AAA GGG AGT GA-3′; Nox-2 fwd: 5′-ACC TTA CTG GCT GGG ATG AA-3′, rev: 5′-TGC AAT GGT CTT GAA CTC GT-3′; Nox-4 fwd: 5′-GAT CAC AGA AGG TCC CTA GCA G-3′, rev: 5′-GTT GAG GGC ATT CAC CAA GT-3′; ANP fwd: 5′- CGT GCC CCG ACC CAC GCC AGC ATG GGC TCC-3′, rev: 5′-GGC TCC GAG GGC CAG CGA GCA GAG CCC TCA-3′; cardiac β-MHC fwd: 5′-CTA CAG GCC TGG GCT TAC CT-3′, rev: 5′-TCT CCT TCT CAG ACT TCC GC-3′; MLC2a: fwd: 5′-TCA GCT GCA TTG ACC AGA AC-3′, rev: 5′-AAG ACG GTG AAG TTG ATG GG-3′-MLC2v: fwd: 5′-AAA GAG GCT CCA GGT CCA AT-3′, rev: 5′-CCT CTC TGC TTG TGT GGT CA-3′. House keeping genes: BACT: fwd: 5′-GAT GAC CCA GAT CAT GTT TGA G-3′, rev: 5′-CCA TCA CAA TGC CTG TGG TA-3′; GAPDH: fwd: 5′-TCG TCC GGT AGA CAA AAT GG-3′, rev: 5′-GAG GTC AAT GAA GGG GTC GT-3′.

Amplifications were performed in an Icycler Optical Module (Biorad, Munich, Germany) using iQ^{TM} SYBR Green Supermix (Biorad). The following programmes were used: Cycle 1, step 1, 95°C for 15 minutes (1×). Cycle 2, step 1, 95°C for 30 seconds (45×). Step 2, specific annealing temperatures for 30 seconds (45×). Step 3, 72°C for 30 seconds (45×). Cycle 3, step 1, 50°C for 10 minutes. Annealing temperatures were: 60°C for Nox-1, GATA-4, ANP, α-MHC, β-MHC, MLC-2c, BACT; 62°C for Nox-2, Nox-4, BMP-10. DTEF-1, MEF2C, Nkx-2.5, α-cardiac actin, MLC2a, GAPDH. C_T values were automatically obtained. Relative expression values were obtained by normalizing C_T values of the tested genes in comparison with C_T values of the housekeeping genes using the $\Delta\Delta$ C_T method.

Measurement of ROS generation

Intracellular ROS levels were measured using the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes, Eugene, OR), which is a nonpolar compound that is converted into a nonfluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. For the experiments, embryoid bodies were incubated in serum-free medium, and 20 μM H_2DCF-DA dissolved in dimethyl sulfoxide (DMSO) was added. After 20 minutes, intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3600 μm^2 regions of interest using an overlay mask unless otherwise indicated. For fluorescence excitation, the 488-nm band of the argon ion laser of the confocal setup was used. Emission was recorded using a longpass LP515-nm filter set.

Statistical analysis

Data are given as mean values ± s.e.m., with *n* denoting the number of experiments unless otherwise indicated. One-way ANOVA for unpaired data was applied as appropriate. A value of $P<0.05$ was considered significant.

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