E2F4 is required for cardiomyocyte proliferation

Machteld J. van Amerongen, Florian Diehl, Tatyana Novoyatleva, Chinmoy Patra, and Felix B. Engel*

Department of Cardiac Development and Remodelling, Excellence Cluster Cardio-Pulmonary System, Max-Planck-Institute for Heart and Lung Research, Parkstrasse 1, Bad Nauheim 61231, Germany

Received 30 June 2009; revised 12 November 2009; accepted 23 November 2009; online publish-ahead-of-print 2 December 2009

Time for primary review: 33 days

Aims
Although the fundamental role of the E2F transcription factor family in cell proliferation is well established, the specific function of E2F4 is unclear. On the basis of findings from cell culture experiments, E2F4 is generally considered as an inhibitor of cell proliferation. Accumulating evidence suggests, however, that E2F4 acts as an activator of cell proliferation in certain contexts. Here, we have investigated the role of E2F4 during heart development and in proliferating cardiomyocytes.

Methods and results
Nuclear E2F4 expression in cardiomyocytes declined during mouse heart development, which correlates with the loss of proliferative capacity of cardiomyocytes. Re-induction of proliferation in postnatal cardiomyocytes increased nuclear E2F4 expression. E2F4 accumulated in the nucleus at the end of the S phase, remained nuclear during mitosis, and disappeared at the end of cytokinesis. siRNA-mediated inhibition of E2F4 in proliferating postnatal cardiomyocytes resulted in a significant reduction in mitosis, but not in DNA synthesis. Co-staining of E2F4 and CreSt revealed that E2F4 co-localizes with kinetochores. Moreover, chromatin immunoprecipitation showed that E2F4 binds to centromeric α-satellite DNA during mitosis.

Conclusion
Our data indicate that E2F4 is required for cardiomyocyte proliferation and suggest a function for E2F4 in mitosis.

Keywords
E2F4 • Cell cycle • Cardiomyocyte proliferation • Kinetochoore

1. Introduction
Proliferation of mammalian cardiomyocytes ceases around birth and is replaced by hypertrophy. The proliferation block prevents efficient tissue regeneration upon injury. Thus, understanding the mechanisms regulating cardiomyocyte proliferation might offer new possibilities to enhance their regenerative capacity.

The role of E2F transcription factors in regulating the cell cycle by activating genes involved in DNA replication and cell-cycle progression is well established. The E2F family comprises eight genes encoding nine proteins (E2F1, E2F2, E2F3a, E2F3b, E2F4, E2F5, E2F6, E2F7, and E2F8). On the basis of structural homology, binding of pocket proteins and their ability to activate transcription when overexpressed, E2F proteins are classified into transcriptional activators (E2F1 to E2F3a) or repressors (E2F3b to E2F8). E2F4 accounts for the majority of E2F4 protein throughout the cell cycle, suggesting a pivotal role for E2F4 in establishing the properties of endogenous E2F activity. The transcriptional activity of E2F4 is regulated by its subcellular localization. During the cell cycle, the ratio of nuclear to cytoplasmic E2F4 changes. In most quiescent cells, E2F4 is primarily nuclear and it localizes to the cytoplasm during entry into the S phase. The localization of E2F4 during G2 phase and mitosis as well as a possible function therein is unclear.

Nuclear E2F4 is mainly bound to p130 in G0 and to retinoblastoma protein (pRb) and p107 during the G1-to-S transition. Although these observations have led to the designation of E2F4 as a ‘repressor’, accumulating evidence suggests that E2F4 can also act as an activator of cell proliferation.

Mice deficient in E2F4 exhibit general growth retardation, demonstrating its importance in development. Specific cardiac defects have not been described. It has been demonstrated that loss of E2F4 impairs proliferation in foetal erythropoiesis and delays the development of Rb mutant tumours. Moreover, E2F4 overexpression can, in certain cell types, reactivate the cell cycle, both in vitro and in vivo. Interestingly, in neonatal cardiomyocytes, E2F4 overexpression has been shown to result in DNA synthesis. Finally, induction of nuclear E2F4 accumulation by the addition of a nuclear localization signal (NLS) enables E2F4 to activate transcription with an efficiency similar to that of the other E2F family members. Taken together, these studies suggest a positive role for E2F4 in cell-cycle activation in a certain context.
However, E2F4 overexpression or long term chronic loss of E2F, as in knockout mice, may not accurately reflect the normal role of E2F4 in vivo. Exogenous E2F4 might compete with inhibitors of endogenous E2Fs, whereas chronic loss might induce compensation mechanisms. Hence, we decided to complement these studies with a model of acute loss of E2F4. Our recently developed assay\textsuperscript{16} made it possible to study endogenous E2F4 employing siRNA technology in postnatal cardiomyocytes that re-enter the cell cycle.

Here, we show that nuclear E2F4 expression in cardiomyocytes declines during development, which correlates with the decline of cardiomyocyte proliferation. In addition, we demonstrate that E2F4 is re-expressed in the nucleus upon induction of cell-cycle re-entry in neonatal cardiomyocytes and that it is required for cell-cycle progression of these cells. Moreover, our data suggest an, to our knowledge, unknown role for E2F4 in mitosis. Taken together, our data show that nuclear E2F4 is required for cell-cycle regulation in mammalian cardiomyocytes.

2. Methods

2.1 Animals and cardiomyocyte isolation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animal experiments were approved by the local committee for Care and Use of Laboratory Animals (Regierungsp"{a}rsidium Darmstadt). Ventricular cardiomyocytes from foetal (embryonic days (E)17), 3-day-old (postnatal days (P)3), and adult (>10 weeks, 200–250 g) Sprague–Dawley rats (Charles Rivers, or own breed) were isolated and cultured as described.\textsuperscript{17,18}

2.2 Stimulation and adenoviruses

Neonatal cardiomyocytes were initially cultured in the presence of 5% horse serum and 20 \( \mu \)g/mL cytosine \( \beta\)-arabinofuranoside (Sigma-Aldrich) before stimulation or virus administration to prevent proliferation of non-cardiomyocytes. After 72 h, the neonatal cardiomyocytes were washed and stimulated once with FGF1 (50 ng/mL, R&D Systems) and stimulated once with FGF1 (50 ng/mL, R&D Systems) added to the culture medium. SB203580\textsuperscript{HCl} (Tocris) was added every day for a total of 3 days. Extracts were briefly sonicated and centrifuged at 17 000 \( \times \) g for 10 min. The supernatant was used as whole-cell extract. Nuclear extracts were obtained by using the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce). Equal amounts of protein homogenates were separated using NuPAGE Novex Bis-Tris Gels (Invitrogen), blotted onto nitrocellulose membranes and analysed (for antibody details, see Supplementary material online, Expanded materials and methods).

2.3 Plasmids

Green fluorescent protein-tagged proliferating cell nuclear antigen (GFP-PCNA) expression plasmids were a kind gift of Cardoso.\textsuperscript{19} PCNA was removed by cleaving the plasmid with specific restriction enzymes, and human E2F4 was cloned into the plasmid. In the final expression plasmid, the fusion protein is driven by the CMV promoter. The fusion protein contains an SV40 NLS at the NH2 terminus followed by an linker (GEGGQGQQGPGRGYAYRS).

2.4 MHC-CycD2 transgenic mouse hearts

The generation of the MHC-CycD2 transgenic line as well as induction of myocardial infarction (MI) was described previously.\textsuperscript{20}

2.5 Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA of mouse cardiac ventricles (E10.5–18.5 \( n \geq 10 \), P0–7, and adult \( n \geq 3 \)) from different developmental stages was isolated using Trizol (Invitrogen). Total RNA of cells was isolated using the RNeasy Mini Kit (Qiagen). RT–PCR was performed following standard protocols (for primers, see Supplementary material online, Expanded materials and methods).

2.6 Western blot

For whole protein lysates, cells or mouse cardiac ventricles were lysed in a lysis buffer (Cell Signaling) with additional 1 mM phenylmethylsulfonyl fluoride and 1 \( \times \) protease inhibitor cocktail (Sigma-Aldrich), for 20 min on ice. Extracts were briefly sonicated and centrifuged at 17 000 \( \times \) g for 4°C for 10 min. The supernatant was used as whole-cell extract. Nuclear extracts were obtained by using the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce). Equal amounts of protein homogenates were separated using NuPAGE Novex Bis-Tris Gels (Invitrogen), blotted onto nitrocellulose membranes and analysed (for antibody details, see Supplementary material online, Expanded materials and methods).

2.7 Immunohistochemistry

Hearts of C57BL/6 mice from different developmental stages [E12, P3, and adult (>10 weeks)] were dissected (\( n \geq 3 \)), were washed in ice-cold PBS, and fixed in ice-cold 4% formaldehyde (overnight), then embedded in paraffin. Hearts of MHC-CycD2 transgenic and non-transgenic mice were snap-frozen in liquid nitrogen. Sections were cut transversally (5–7 \( \mu \)m). Cultured cells were fixed in 3.7% formaldehyde for 15 min. Immunohistochemistry was performed following standard protocols (for details and antibodies, see Supplementary material online, Expanded materials and methods).

2.8 RNA interference

For siRNA-mediated gene knockdown in Rat 2 fibroblasts (ATCC), fibroblasts were grown to 60–70% confluence and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For siRNA-mediated gene knockdown in cardiomyocytes, siRNA was electroporated into freshly isolated neonatal rat cardiomyocytes according to the manufacturer’s instructions (Amaxa). siRNAs were designed and synthesized by Qiagen against E2F4 mRNA (E2F4_1: 5′-AACGAATGAATTTCCCTATATAA-3′) or a different region of the E2F4 mRNA sequence (E2F4_2: 5′-TAGAGCCATTGCAGAGATTTA-3′). AllStars Negative Control siRNA (Qiagen), a validated non-silencing siRNA, was used as a negative control.

2.9 Chromatin immunoprecipitation

For chromatin immunoprecipitation (ChIP), a ChIP Assay Kit was used according to the manufacturer's instructions (Millipore) (for details and primers, see Supplementary material online, Expanded materials and methods).

2.10 Statistical analysis

For immunofluorescence analyses, 400–1000 cardiomyocyte nuclei were counted. Data are expressed as mean \( \pm \) SEM of at least three independent experiments. The data were analysed using GraphPad Prism (version 4.00, GraphPad Software, Inc.). The statistical significance of differences was evaluated by a Student's t-test. A difference of \( P < 0.05 \) was considered statistically significant.
3. Results

3.1 Nuclear E2F4 expression disappears during heart development

To determine the expression of E2F4 during heart development, we collected mouse cardiac ventricles at sequential developmental stages (E10.5–18.5, P0–7, and adult) and determined the relative mRNA and protein expression of E2F4 at these time points. As seen in Figure 1A and B, both E2F4 mRNA and protein were highly expressed in embryonic mouse cardiac ventricles and their expression gradually decreased as development progressed. Similar data were obtained in rat (data not shown). It is known that E2F4 can be localized either in the cytoplasm or in the nucleus, whereas nuclear localization is, self-evidently, required for its transcriptional activity. Therefore, we analysed nuclear localization of E2F4 in isolated rat ventricular cardiomyocytes from different developmental stages by immunohistochemistry (Figure 1C). Strong nuclear E2F4 staining was observed in foetal (E17.5) cardiomyocytes (21.0 ± 2%), whereas E2F4 staining was rarely observed in the nuclei of neonatal (P3) cardiomyocytes (1.9 ± 0.6%) and never in the nuclei of adult (12 weeks) cardiomyocytes (>6000 cardiomyocytes analysed). The specificity of the E2F4 antibody [E2F4 (C20)] used in this study was confirmed by western blot analysis using protein extracts of wild-type and E2F4−/− mouse

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/86/1/92/307233/Downloaded from https://academic.oup.com/cardiovascres/article-abstract/86/1/92/307233 by guest on 05 June 2018)

Figure 1 Nuclear E2F4 expression declines during heart development. (A) RT–PCR analysis of E2F4 mRNA expression and (B) western blot analysis of E2F4 protein expression during heart development. mRNA and protein were isolated from mouse cardiac ventricles collected at sequential developmental stages (embryonic days 10.5–18.5, E10.5–18.5, postnatal days 0–7, P0–7, and adult). Equal amounts were loaded as verified by using GAPDH as a loading control. (C) Isolated foetal (E17.5), neonatal (P3), and adult (12 weeks) rat ventricular cardiomyocytes stained for E2F4 (green) and the cardiomyocyte-specific marker sarcomeric α-actinin (red). Nuclei are stained by DAPI (blue). Magnification × 200. (D) Western blot analysis of E2F4 protein expression in protein extracts from non-transgenic (non-txg) and MHC-CycD2 transgenic mouse cardiac ventricles. GAPDH is shown as loading control. (E) Examples of cardiomyocytes expressing nuclear E2F4 in MHC-CycD2 transgenic hearts 7 days after MI stained for E2F4 (red) and the cardiomyocyte-specific marker caveolin 3 (green). Nuclei are stained by DAPI (blue). Arrows indicate E2F4-stained cardiomyocyte nuclei. Arrowheads indicate E2F4-stained non-cardiomyocyte nuclei. Magnification × 400.
embryonic fibroblasts isolated from E13.5-day-old embryos\(^8\) and recombinant E2F4 (Panomics) and by immunofluorescence staining of sections from wild-type and E2F4\(^{-/-}\) mice\(^7\) (see Supplementary material online, Figure S1). On sections, the antibody gave a slight background staining, but specific nuclear E2F4 staining could easily be distinguished. A similar nuclear E2F4 expression pattern was observed in paraffin sections of the ventricular myocardium from foetal, neonatal, and adult hearts stained for E2F4, which were stained using an E2F4 antibody suitable for paraffin (TFE-42; see Supplementary material online, Figure S2). Taken together, the nuclear E2F4 localization in foetal cardiomyocytes and the decline of cardiomyocytes expressing nuclear E2F4 as development progresses suggest a correlation between nuclear E2F4 expression and cardiomyocyte proliferation that declines also during development and ceases shortly after birth.

In order to support a correlation between cardiomyocyte proliferation and E2F4 expression, we analysed transgenic mice expressing cyclin D2 under the regulation of the \(\alpha\)-cardiac myosin heavy chain (MHC) promoter (designated MHC-CycD2 mice).\(^2\) Postnatal cardiomyocytes of these mice exhibit an increased level of cell-cycle activity under baseline conditions and following MI. Western blot analysis of protein extracts of uninjured MHC-CycD2 and non-transgenic adult mouse cardiac ventricles demonstrated that E2F4 expression was higher in MHC-CycD2 hearts compared with non-transgenic hearts (Figure 1D). Moreover, co-staining of heart sections of MHC-CycD2 and non-transgenic mice for E2F4 and the cardiomyocyte-specific marker caveolin 3 at 7 days after MI demonstrated the presence of nuclear E2F4 in cardiomyocytes in the peri-infarct area of the MHC-CycD2 mice (Figure 1E), which was not observed in cardiomyocytes of non-transgenic mice. These data support the notion that nuclear E2F4 expression is associated with cardiomyocyte proliferation.

### 3.2 Cell-cycle re-entry in neonatal cardiomyocytes causes expression of nuclear E2F4

To further strengthen the assumption that nuclear E2F4 is associated with cardiomyocyte proliferation, we tested whether E2F4 is expressed in the nucleus upon induction of cell-cycle re-entry in neonatal cardiomyocytes. We used our recently developed assay in which one can reverse cell-cycle arrest in cardiomyocytes.\(^2\) We stimulated neonatal rat cardiomyocytes with FGF1 and a p38 MAP kinase inhibitor, SB203580 (p38i), for 3 days to induce cell-cycle re-entry and assessed the expression pattern of E2F4. FGF1/p38i stimulation increased the number of BrdU (5-bromo-2'-deoxyuridine, marker for DNA synthesis, present during the last 24 h of culturing)-positive nuclei compared with the control 72 h after stimulation (E2F4 siRNA 1.7\% vs. control 0.6\%; \(P < 0.05\)) (Figure 3D). Moreover, co-staining of heart sections of MHC-CycD2 and non-transgenic mice for E2F4 and the cardiomyocyte-specific marker caveolin 3 at 7 days after MI demonstrated the presence of nuclear E2F4 in cardiomyocytes in the peri-infarct area of the MHC-CycD2 mice (Figure 1E), which was not observed in cardiomyocytes of non-transgenic mice. These data support the notion that nuclear E2F4 expression is associated with cardiomyocyte proliferation.

3.3 E2F4 is required for cell-cycle progression in cardiomyocytes

To directly determine whether E2F4 is required for cardiomyocyte proliferation, we performed knockdown experiments using RNA interference. The specificity of siRNA-mediated knockdown was tested in Rat 2 fibroblasts. siRNA transfection using two different E2F4 siRNAs suppressed E2F4 mRNA expression but not E2F1, E2F3, or E2F5 mRNA expression and downregulated E2F4 protein as well (Figure 3A and 8). E2F2 is not annotated in the rat genome and also an NCBI homologene search did not reveal a sequence for rat E2F2, therefore we could not demonstrate the effect of E2F4 siRNA on the expression of E2F2 in rat cells. Next, we electroporated siRNA into neonatal cardiomyocytes directly after isolation. Forty-eight hours after isolation, cardiomyocytes were stimulated with FGF1/p38i to induce cell-cycle re-entry for 2 or 3 days (until 96 or 120 h after isolation, Figure 3C). siRNA transfection resulted in downregulation of E2F4 protein (E2F4_1 siRNA 44\% \(\pm\) 14\% vs. E2F4_2 siRNA 61\% \(\pm\) 12\%; \(n = 3\); \(P < 0.05\), Figure 3D). E2F4 was still downregulated 96 h after isolation (Figure 3D). To analyse the effect of E2F4 knockdown on cardiomyocyte proliferation, we counted BrdU and H3P (phosphorylated histone H3, marker for late G2 and M phase)/sarcomeric \(\alpha\)-actinin-positive cardiomyocytes. The cells were labelled with BrdU 24 to 48 h after the start of FGF1/p38i stimulation. Suppression of E2F4 by siRNA did not alter the number of BrdU-positive cardiomyocytes 48 h after stimulation (Figure 3E). However, E2F4 knockdown significantly reduced the number of H3P-positive cardiomyocytes nuclei after FGF1/p38i stimulation at this time (E2F4 siRNA 1.8\% \(\pm\) 0.44\% vs. control siRNA 4.3\% \(\pm\) 1.26\%; \(n = 3\); \(P < 0.05\), Figure 3F). As these data indicate that downregulation of E2F4 inhibits mitosis in cardiomyocytes, we also assessed the number of BrdU-positive cardiomyocytes 72 h after stimulation, that is, after the cells (and nuclei) have divided. Downregulation of E2F4 resulted in a significant decreased number of BrdU-positive nuclei compared with the control 72 h after stimulation (E2F4 siRNA 42.5\% \(\pm\) 3.3\% vs. control siRNA 54.5\% \(\pm\) 2.1\%; \(n = 3\); \(P < 0.05\), Figure 3E). These data demonstrate that E2F4 is required for cardiomyocyte proliferation and that late G2 and M phases are primarily affected by the knockdown of E2F4.

3.4 E2F2 but not E2F4 overexpression stimulates cell-cycle re-entry of cardiomyocytes

To determine whether E2F4 is sufficient to induce cardiomyocyte proliferation, we performed overexpression experiments. Adenoviruses expressing E2F2, E2F4, and LacZ as a control were a kind gift from Braun.\(^1\) As shown in Figure 4A and 8, overexpression of E2F2, but not E2F4, significantly increased the number of BrdU-positive cardiomyocytes compared with LacZ control virus (adE2F2 80.8\% \(\pm\) 1.6\% vs. adLacZ 3.5\% \(\pm\) 1.7\%; \(n = 3\); \(P < 0.001\), Figure 4B).
Using immunohistological staining, strong nuclear staining for E2F2 was observed in cardiomyocytes after overexpression of E2F2 (Figure 4C), whereas strong cytoplasmic staining for E2F4 was observed in cardiomyocytes after overexpression of E2F4 (Figure 4D).

As our data suggest that E2F4 should be expressed in the nucleus to trigger cardiomyocyte proliferation, we overexpressed NLS-tagged E2F4 in freshly isolated neonatal cardiomyocytes. Seventy-two hours after isolation, cardiomyocytes were stimulated with FGF1/p38i to induce cell-cycle re-entry for 3 days or were kept unstimulated. During the culture period, NLS-GFP-E2F4-expressing cardiomyocytes demonstrated typical features of apoptosis; they stopped contracting, demonstrated cytoplasmic shrinkage, and detached from the culture plate (data not shown). Taken together, these data demonstrate that overexpression of E2F4 does not lead to the S-phase re-entry of neonatal (P3) rat cardiomyocytes, but rather to cell death.

3.5 E2F4 is expressed in the nucleus during cardiomyocyte mitosis

To determine whether nuclear E2F4 is expressed at a particular cell-cycle phase, we co-stained FGF1/p38i-stimulated neonatal cardiomyocytes with E2F4 and cell-cycle-phase-specific markers. Short-term
BrdU labelling (1 h) was used to identify the cardiomyocytes going through the S phase. Co-staining of BrdU and H3P confirmed that BrdU-labelled cells had not yet proceeded to G2/M phase, because they were negative for H3P (data not shown). Co-staining of E2F4 and BrdU showed that BrdU-positive cells were often negative for nuclear E2F4 (Figure 5A). In contrast, H3P-positive cells were always positive for nuclear or chromosome-associated E2F4 (Figure 5B), whereas E2F1 was always absent (see Supplementary material online, Figure S3). E2F4 seemed to localize on the chromosomes, exhibiting a ‘dotty’ staining pattern in prophase nuclei. Co-staining of E2F4 and sarcomeric α-actinin confirmed the cardiomyocyte identity of these cells (see Supplementary material online, Figure S4). Co-staining of E2F4 and Crest, an anti-kinetochore antibody, indicated using confocal microscopy that E2F4 is localized at the kinetochores, which mediate the binding of spindle microtubules to chromosomes during mitosis (Figure 5C). This staining pattern was also observed in foetal cardiomyocytes and cardiac fibroblasts (see Supplementary material online, Figure S5). Aurora B staining confirmed that E2F4 was localized to the chromosomes of all anaphase cells (Aurora B is located at the mid-zone). However, at the end of cytokinesis when the mid-body (Aurora B) was present, nuclear E2F4 was only infrequently observed (Figure 5D). Similar results were obtained by co-staining of foetal (proliferating) cardiomyocytes and cell-cycle-phase-specific markers (data not shown). This suggests that nuclear E2F4 becomes expressed in

**Figure 3** E2F4 is required for cell-cycle progression in cardiomyocytes. (A and B) Rat 2 fibroblasts were transfected with two different siRNAs against E2F4 (1 and 2) or a validated non-silencing negative control siRNA. (A) RT–PCR analysis of E2F1, 3, 4, and 5 mRNA expression 48 h after transfection demonstrates the specificity of the siRNAs towards E2F4. Representative gels are shown. GAPDH is shown as loading control. (B) Western blot analysis of E2F4 expression in Rat 2 fibroblasts 48 h after transfection shows the efficient knockdown of E2F4 protein. (C–E) Neonatal cardiomyocytes were transfected with two different siRNAs against E2F4 (1 and 2) or a validated non-silencing siRNA directly after isolation. Forty-eight hours after cardiomyocyte isolation, cardiomyocytes were stimulated with FGF1/p38i for 2 or 3 days (until 96 or 120 h after isolation). Forty-eight hours and 96 h after cardiomyocyte isolation, E2F4 knockdown was verified by western blot analysis. (C) Schematic overview of the E2F4 knockdown experiments utilizing RNA interference in cardiomyocytes. (D) Western blot analysis of E2F4 expression during FGF1/p38i stimulation of neonatal cardiomyocytes. GAPDH is shown as loading control. (E) Quantitative analysis of BrdU expression in cardiomyocytes transfected with siRNA against E2F4 (2) or negative control siRNA 48 and 72 h after FGF1/p38i stimulation. Bars represent mean ± SEM, n = 3. (F) Quantitative analysis of H3P expression in cardiomyocytes transfected with siRNA against E2F4 (2) or negative control siRNA 48 h after FGF1/p38i stimulation. Significant difference compared with negative control is indicated as *P < 0.05. Bars represent mean ± SEM, n = 3.
the nucleus at the end of the S phase, locates to the chromosomes during G2/M phase, and finally disappears from the nucleus at the end of cytokinesis. Taken together, these data indicate that E2F4 plays a role in cardiomyocyte mitosis and binds to the kinetochores.

3.6 E2F4 binds to the kinetochore region during mitosis

To further support our hypothesis that E2F4 is a kinetochore-associated protein, we performed ChIP to confirm that E2F4 binds to centromeric α-satellite DNA during mitosis, the region where kinetochores are located. The dotty-like expression pattern of E2F4 during prophase was also observed in fibroblasts and thus does not appear to be cardiomyocyte-specific (see Supplementary material online, Figure S5). Therefore, we decided to use HeLa cells to be able to utilize published degenerate primer pairs that recognize human centromeric α-satellite DNA,22,23 as there are no primers known to detect rat centromeric α-satellite DNA. The cross-linked genomic DNA of HeLa cells was sheared into 100–1000 bp fragments, as demonstrated by separation of sheared and unsheared samples on a 2% agarose gel (Figure 6A). PCR using the degenerate primers of genomic DNA of HeLa cells resulted as expected in a ladder of DNA bands with multiples of 171 bp of length,23 which consist of the centromeric DNA repeat units (Figure 6B). Immunoprecipitation of E2F4, using two different E2F4 antibodies (C20 or A20), was confirmed by western blot (Figure 6C). Finally, ChIP was performed using the C20 against E2F4 or rabbit IgG as control followed by PCR using the primer pairs against centromeric α-satellite repeats or a known E2F4 target, the Kif2C promoter.24 A representative result using E2F4 (C20) is shown (n = 3, Figure 6D). The E2F4 (A20) antibody gave comparable results (data not shown). The results demonstrate that E2F4 associates with centromeric α-satellite DNA and further supports our hypothesis that E2F4 is a kinetochore-associated protein.

4. Discussion

We conclude from our study that endogenous E2F4 plays a positive role in cardiomyocyte proliferation. Our data suggest that E2F4 has, to our knowledge, so far unknown function during mitosis and is a kinetochore-associated protein. Several lines of evidence support these conclusions. First, nuclear E2F4 expression is developmentally regulated; nuclear E2F4 expression in cardiomyocytes declines during heart development that correlates with their loss of proliferative capacity. Secondly, induction of cell-cycle re-entry of postnatal cardiomyocytes re-induces expression of nuclear E2F4. Progression into mitosis is blocked by siRNA-mediated E2F4 knockdown.
Figure 5 E2F4 is expressed in the nucleus during cardiomyocyte mitosis. (A) FGF1/p38i-stimulated cardiomyocytes stained for E2F4 (green), BrdU (red), and DAPI (blue). Nuclei are stained by DAPI (blue). Cardiomyocytes were pulse labelled with BrdU for 1 h. Arrows indicate E2F4-positive cells that are BrdU-negative. Arrowheads indicate BrdU-positive cells that are E2F4-negative. Note that mitotic cell (*) is BrdU-negative. Magnification ×200. (B) FGF1/p38i-stimulated cardiomyocytes stained for E2F4 (green), H3P (red), and DNA (DAPI, blue). Note the ‘dotty’ E2F4-staining pattern in prophase. Magnification ×400. (C) Confocal image of an FGF1/p38i-stimulated cardiomyocyte stained for E2F4 (green) and the kinetochore marker Crest (red). Magnification ×400. (D) FGF1/p38i-stimulated cardiomyocytes stained for E2F4 (green) and Aurora B (red). Magnification ×400.
Thirdly, E2F4 localizes during mitosis to the chromosomes and binds to the kinetochore region.

Previous investigations of E2F4 expression during heart development resulted in controversial data. Our study agrees with previous reports demonstrating that E2F4 expression declines during heart development. We analysed E2F4 expression on mRNA, protein, and cellular level. Most importantly, we concentrated our analysis on the expression of nuclear E2F4 during heart development, which has, to our knowledge, so far not been described. This is important as E2F4 can be localized either to the cytoplasm or to the nucleus.5

Taken together, our data suggest a correlation between nuclear E2F4 expression and cardiomyocyte proliferation that also declines during development and ceases shortly after birth.29 This is further supported by the demonstration that nuclear E2F4 is present in cyclin D2-expressing cardiomyocytes. It has been demonstrated that cardiomyocytes of MHC-CycD2 mice exhibit increased levels of cell-cycle activity under baseline conditions and following MI.20 However, the amount of cardiomyocytes in G2 phase and mitosis in these mice is very small and we observed accordingly only few cardiomyocytes with strong nuclear E2F4 expression.

Endogenous E2F4 was detected in the nucleus of proliferating cardiomyocytes. This is in contrast to previous in vitro studies using cell lines that found that endogenous E2F4 is located only in the nucleus of quiescent cells. During G1- to S-phase transition, the majority of E2F4 localizes to the cytoplasm in these cells.6 Very little free E2F4 is detected in the nucleus.6 However, our results agree with Deschenes et al., who found a correlation between endogenous nuclear E2F4 expression and Ki67 positivity in primary human intestinal epithelial cells. Recently, they demonstrated that knockdown of E2F4 decreases the proliferation rate of these cells.31 This suggests a possible discrepancy between E2F4 function in cell lines and primary cells. In addition, the observation of nuclear E2F4 during proliferation indicates the requisite binding of another protein to E2F4 providing a heterologous NLS, because E2F4 lacks an NLS.4,5

Although E2F4-deficient mice exhibit general growth retardation and impaired cellular proliferation in foetal erythropoiesis, a specific cardiac defect has not been described. Here we show that transient siRNA-mediated knockdown of E2F4 significantly reduces the number of mitotic cardiomyocytes, but not the S-phase cardiomyocytes, which demonstrates that E2F4 is required for cardiomyocyte proliferation and that late G2 and M phases are primarily affected by the knockdown of E2F4.

Lately, more and more evidence has accumulated questioning the assumption that E2F4 is exclusively a negative regulator of cell-cycle progression. E2F4 overexpression can reactivate the cell cycle, both in vitro and in vivo.14 Interestingly, Ebelt et al. showed that overexpression of E2F4 induces the S-phase re-entry in isolated neonatal cardiomyocytes, although the number of BrdU-positive cardiomyocytes was less than after E2F2 overexpression. In our hands, E2F2 overexpression resulted in the S-phase re-entry of neonatal cardiomyocytes as well. However, we did not observe the S-phase re-entry upon overexpression of E2F4. At the same time, we observed that E2F2, which contains an NLS, localized to the nucleus, but that E2F4, lacking an NLS, localized to the cytoplasm. Other studies demonstrated as well that E2F4 accumulates in the cytoplasm after overexpression, unless a heterologous NLS was provided.4,5 Nevertheless, simply overexpressing an NLS-tagged E2F4 did not lead to induction of cardiomyocyte proliferation but to cell death.
This finding is in agreement with Garneau et al.32 who showed that overexpression of nuclear E2F4 induces apoptosis in normal intestinal crypt cells. They suggested that induction of apoptosis might be a protection mechanism against tumour formation. Interestingly, the E2F4 gene contains a serine repeat trinucleotide (AGC), that is frequently mutated in various tumours.33 One could speculate that E2F4 mutants could be created that have lost the capacity to trigger apoptosis, thereby inducing cardiomyocyte proliferation.

The difference between the study of Ebel et al. and ours might lay in the fact that they isolated cardiomyocytes from newborn 0-day-old (P0) rats and we isolated cardiomyocytes from 3-day-old (P3) rats. In P0 cardiomyocytes, factors that can bind E2F4 and provide an NLS might be still available and are downregulated shortly after P0, and a protection mechanism against cardiomyocyte proliferation might not have been activated yet. This is supported by the fact that Ebel et al. recently demonstrated that overexpression of E2F4 in the heart of healthy adult mice using an adenoviral vector does not result in the S-phase re-entry.34 In addition, overexpression of E2F4 might result in increased pocket protein binding to E2F4 and thus in the release of the ‘activator’ E2Fs. For example, E2F1, which is inactivated in neonatal cardiomyocytes15 (see Supplementary material online, Figure S3), is inactivated by the binding of p130 and pRB in neonatal cardiomyocytes.36 Overexpression of E2F1 results in cell-cycle activation.35,37 Thus, it is important to use alternative strategies such as siRNA-mediated knockdown to elucidate the role of E2F4 in cardiomyocyte proliferation.

Surprisingly, our data suggest that E2F4 is a kinetochore-associated protein. E2F4 is known to act as a transcription factor. Therefore, it is possible that E2F4 regulates the expression of genes that are closely located to the kinetochore. However, due to its co-localization with Crest, our data might rather indicate that E2F4 has another function. The kinetochore is a large protein assembly around the centromere that mediates the attachment of chromosomes to spindle microtubules coordinating chromosome segregation.38 Our data suggest that E2F4 can function as a kinetochore protein during mitosis and warrant additional future experiments to determine its function.

Understanding the mechanisms that regulate cardiomyocyte proliferation is important, as it has been shown that induction of cardiomyocyte proliferation is a potential future option for cardiac regeneration.16,20,21 It has been demonstrated that many approaches can induce DNA synthesis in the majority of postnatal cardiomyocytes. However, most of the cells fail to undergo mitosis and cell division.39 Thus it is important to better understand how cell-cycle progression beyond the S phase (DNA synthesis) is regulated. Here we show that E2F4 is expressed during mitosis in normal proliferating foetal cardiomyocytes as well as after induction of proliferation in postnatal cells. We demonstrate that it is required for mitosis and provided evidence for an unknown function of E2F4 as a kinetochore-associated protein. Moreover, we demonstrate that E2F4 expression is maintained in cyclin D2 overexpressing mice that have the ability to regenerate heart after MI. Taken together, we have established E2F4 as a required regulator of cardiomyocyte proliferation, opening up new avenues to study the regulation of cell-cycle control in cardiomyocytes, which might be a starting point for novel strategies for cardiac repair.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We are grateful to Ingrid Hauck-Schmalenberger for technical support, L. Field for MHC-CycD2 transgenic and non-transgenic hearts, J. Nevins for MEFS, R. Slack for sections of E2F4+/− and E2F4+−/− mouse embryos, W. Earnshaw for Crest autimmune serum, M.C. Cardoso for the PCNA-GFP plasmid, and T. Braun for E2F and β-galactosidase expressing adenoviruses and critical reading of the manuscript.

Conflict of interest: none declared.

Funding

This work was supported by the Alexander von Humboldt Foundation (Bonn, Germany, Goethe Universität, Gotha, to E.F.B., to J.N. and M.J.A.); the Excellence Cluster Cardio-Pulmonary System (Giessen, Germany, to R.D., to T.N., and to M.J.A.).

References


13. Gill RM, Hamel PA. Subcellular compartmentalization of E2F family members is cell cycle-dependent.


