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Characterisation and regulation of E2F-6 and E2F-6b in the rat heart: a potential target for myocardial regeneration?

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Abstract

The E2F transcription factors are instrumental in regulating cell cycle progression and growth, including that in cardiomyocytes, which exit the cell cycle shortly after birth. E2F-6 has been demonstrated to act as a transcriptional repressor; however, its potential role in normal cardiomyocyte proliferation and hypertrophy has not previously been investigated. Here we report the isolation and characterisation of E2F-6 and E2F-6b in rat cardiomyocytes and consider its potential as a target for myocardial regeneration following injury. At the mRNA level, both rat E2F-6 and the alternatively spliced variant, E2F-6b, were expressed in E18 myocytes and levels were maintained throughout development into adulthood. Interestingly, E2F-6 protein expression was down-regulated during myocyte development suggesting that it is regulated post-transcriptionally in these cells. During myocyte hypertrophy, the mRNA expressions of E2F-6 and E2F-6b were not regulated whereas E2F-6 protein was up-regulated significantly. Indeed, E2F-6 protein expression levels closely parallel the developmental withdrawal of myocytes from the cell cycle and the subsequent re-activation of their cell cycle machinery during hypertrophic growth. Furthermore, depletion of E2F-6, using anti-sense technology, results in death of cultured neonatal myocytes. Taken together, abrogation of E2F-6 expression in neonatal cardiomyocytes leads to a significant decrease in their viability, consistent with the notion that E2F-6 might be required for maintaining normal myocyte growth.

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Introduction

The ability of mammalian cardiomyocytes to undergo hyperplastic growth decreases significantly during development such that the vast majority of adult myocytes are unable to divide; instead they grow by hypertrophy (Bicknell et al 2003). This terminal differentiation of cardiomyocytes restricts the ability of the adult heart to regenerate new myocardial tissue following injury (e.g. myocardial infarction (MI)). Additionally, prolonged haemodynamic stress on the heart (e.g., as a result of chronic hypertension) can lead to myocardial adaptation and the development of cardiac hypertrophy. The switch from hyperplastic to hypertrophic growth in mammalian cardiomyocytes has been attributed to a block in their cell cycle machinery (Li et al 1996; Poolman & Brooks 1998; Bicknell et al 2003) such that the majority of adult myocytes are blocked in the G_0/G_1 phases of the cycle, whereas hypertrophic growth drives a significant proportion of these cells into S and G_2/M, although additional checkpoints prohibit them from undergoing cytokinesis (Capasso et al 1992; Li et al 1998; Bicknell et al 2003).

The E2F family of transcription factors comprises at least 7 distinct members, E2Fs 1–7, all of which require heterodimerisation with a related DP subunit (DP-1 or DP-2) for full activity (Dyson 1998). Apart from E2F-7 (de Bruin et al 2003), all members share strong homology in their heterodimerisation and DNA-binding domains, their marked box, and, with the exception of E2F-6 and E2F-7, a transactivation domain and a pocket protein-binding region that resides within this sequence (Dyson 1998). E2F transcription factors are divided into three main groups: A, E2F-1, E2F-2 and E2F-3a, which play a role in progression from G_1 into S-phase of the cell cycle and possess a pRb binding site within their transactivation domain; B, E2F-4 and E2F-5, which bind to p107 or p130 members of the pocket proteins family and E2F-3b, which plays a role in differentiation and proliferation; and C,

E2F-6 and E2F-7, which are unique since they lack both transactivation and pocket protein-binding domains.

There is ample evidence suggesting that E2F-6 functions as a repressor of transcription during G_0 and during S-phase in many cell types. We have isolated for the first time rat E2F-6 from heart, investigated its expression and regulation during rat cardiomyocyte development and hypertrophy and evaluated its role in myocyte growth and cell cycle exit. Interestingly, we also independently identified an alternatively spliced isoform of E2F-6, termed E2F-6b, in rat myocytes. In contrast to a role as a repressor of cell cycle progression, our results suggest that the expression of E2F-6 in the rat heart correlates with the exit and re-entry of myocytes from the cell cycle, indicating a role in the G_1/S phases of the cycle in rat cardiomyocytes. Furthermore, abrogation of expression of E2F-6 in neonatal myocytes led to a significant decrease in myocyte viability and suggests that E2F-6 might be required for maintaining normal myocyte growth, thereby identifying this molecule as a potential target for encouraging myocardial regeneration post-MI.

Materials and Methods

Animals and isolation of cardiomyocytes

Adult male Wistar rats, neonatal pups at 2 (P2) or 3 (P3) days after birth and time-mated pregnant mothers (at 18 days gestation; E18) were sacrificed in accordance with UK Home Office approved protocols. Fetal (E18), neonatal (P2/P3) or adult myocytes were isolated from excised ventricular tissues as previously described (Brooks et al 1997).

In-vitro model of hypertrophy

Isolated neonatal myocytes were cultured at a density of 2.5×10^6 per 10 cm^2 Primaria dish and incubated with 5% fetal calf serum (FCS) myocyte media (DMEM:M199 (4:1 v/v) containing 5% v/v FCS and 100 $\mu g\,mL^{-1}$ penicillin/streptomycin; Gibco, UK). After 24 h of incubation, plates were washed twice with phosphate buffered-saline (PBS) and serum starved for 48 h in myocyte media lacking FCS. Myocytes then were treated with FCS-free myocyte media containing one of the following hypertrophic agonists: 20% v/v FCS, phenylephrine (PE; 100 μM) or endothelin-1 (ET-1; 10 nM). Hypertrophic growth was confirmed by a combination of changes in myocyte size, protein content and mRNA expression of hypertrophic marker genes, as described previously (Vara et al 2003).

RNA isolation, semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) and real-time quantitative PCR

Total RNA was purified from $2.5–10\times10^6$ myocytes using TRI-reagent as recommended by the manufacturer (Sigma, UK). Complementary DNA was synthesised from 5 μg of total RNA, as described previously (Vara et al 2003). RT-PCR conditions were as follows: initial denaturation step of 94°C for 2 min followed by a non-saturating number of cycles (as stated below) of 94°C for 30 s, annealing step at 63°C (GAPDH or E2F-6/product 2/E2F-6b) or 65°C (E2F-6 specific or E2F-6b specific) for 30 s, followed by 72°C for 1 min and completed with a final extension cycle of 72°C for 10 min. For each primer pair, cycle curves were performed to determine the optimal number of PCR cycles required to achieve product under non-saturating conditions. Integrity of cDNA was confirmed by amplification (23 cycles) of the house-keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using GAPDH sense (5′ CCTTCATTGACCTCAAC 3′) and GAPDH antisense (5′ AGTTGTCATGGATGACC 3′) primers. E2F-6, product 2 and E2F-6b were amplified in 30 cycles using sense primer MM3 (5′ AGACCCCATCAACGTGGA 3′) and antisense primer MM4 (5′ TCTCTGGGAGCTGGAACATC 3′). E2F-6 was amplified specifically in 30 cycles using sense primer GB91 (5′ TGGAGAACCTACTGCCATC 3′) and antisense primer MM4. E2F-6b was amplified specifically in 30 cycles using sense primer GB92 (5′ TTCTAGCCAGGTGTGGTGGC 3′) and antisense primer MM4. RT-PCR products were separated on 2% agarose gels containing 0.5 $\mu g\,mL^{-1}$ of ethidium bromide and were viewed and photographed under ultraviolet light.

Real-time quantitative PCR analysis was performed on a ABI 5700 sequence detection system using Absolute QPCR SYBR Green ROX mix (ABgene). Rat E2F-6 mRNA expression was quantified using sense primer GB91mod (5′ CGTGGAGAACCTACTGCCATCA 3′) and antisense primer GB150 (5′ CAGCGACACATCAAACCGG 3′). Rat E2F-6b was amplified using sense primer GB93 (5′ GGAGGCAGAGGCAGAAGGA 3′) and antisense primer GB150. The specificity of amplifications was assessed using a temperature dissociation curve. E2F-6 mRNA expressions were normalised to GAPDH mRNA expression using the rodent GAPDH control reagent kit (Applied Biosystems Inc) as recommended by the manufacturer.

Protein extraction, protein determination, SDS-PAGE and Western blotting

Protein lysates were prepared from E18, P2 and adult myocytes and their concentrations determined using a modified Bradford procedure using BSA type V (Sigma, UK) as a standard, as described previously (Brooks et al 1997). Proteins were separated electrophoretically on 14% sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gels and transferred to nitrocellulose membranes according to established methods (Brooks et al 1997). E2F-6 was detected using a mouse E2F-6 antibody directed against full-length human E2F-6 (1:20 dilution; a kind gift from Drs Trimarchi and Lees, MIT, USA) and an appropriate horseradish peroxidase-conjugated secondary antibody (1:4000 dilution; DAKO, UK) using standard methods. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL; Amersham, UK).

S1 nuclease treatment

RT-PCR was performed on cDNA synthesised from E18 myocytes, using MM3 and MM4 primers as described above. RT-PCR products were purified using a PCR Purification Kit (Qiagen, UK), as described by the manufacturer, and DNA was eluted in water. To 0.5 μg of purified DNA, S1 nuclease buffer was added to a final concentration of 1×, and either 5

units of S1 nuclease enzyme or an equal volume of 1×S1 nuclease buffer was added. Both samples were incubated at 37°C for 30 min before separating on a 2% agarose/TAE gel containing 0.5 μg mL^{-1} ethidium bromide.

Constructs and myocyte transfection

The full ORF sequence of human E2F-6 (a generous gift from Dr Cartwright, European Institute of Oncology, Milan, Italy) was sub-cloned in both sense and anti-sense orientations into pAG-IRES-EGFP, an expression vector generated by replacing the cytomegalovirus (CMV) promoter cassette of pIRES2-EGFP (Clontech, BD Biosciences, UK) with the pAG promoter cassette (Miyazaki et al 1989) from the pCAGGS vector (Niwa et al 1991).

For immunocytochemical studies, sterile glass coverslips (13 mm^2) were coated with sterile 1% w/v gelatin solution. Myocytes (5×10^4) were added to each well containing the gelatin-coated coverslip and cultured for 24 h in 5% FCS myocyte media (without BrdU). For Western analysis, 2.5×10^5 myocytes were cultured in 6-well plates as described above. Transfections were performed using 4 μL of GeneJuice transfection reagent (Novagen, UK) with 0.5 μg of expression construct DNA, pAG-IRES-EGFP (control), E2F-6 sense or E2F-6 anti-sense, as recommended by the manufacturer. Transfected cultures were incubated for 48 h before analysis. Transfection efficiency was determined by monitoring EGFP expression in transfected cultures and was estimated to be about 25–30% (data not shown).

Immunocytochemical analysis

Myocytes were fixed with 1% formaldehyde in PBS and incubated at room temperature for 10 min, and subsequently permeabilised with 0.1% Triton X100/PBS solution for 15 min at room temperature with gentle agitation. Fixed cells then were blocked with 200 μL of 10% goat serum–2% BSA–PBS for 30 min at room temperature with gentle agitation. Following removal of the blocking solution, cells were washed with 1 mL of PBS for 5 min before addition of mouse α-sarcomeric tropomyosin monoclonal antibody (1:100 dilution; Sigma, UK) in 200 μL of 1% goat serum–2% BSA–PBS for 60 min at room temperature with gentle agitation. Myocytes then were washed thoroughly with PBS before addition of the Alexa Fluor-568 goat anti-mouse secondary antibody (1:100 dilution; Molecular Probes Europe BV, The Netherlands) in 200 μL of 1% rabbit serum–2% BSA–PBS for 45 min at room temperature with gentle agitation. Finally, coverslips were washed with PBS before being mounted using Vectashield with DAPI (Vector Laboratories, UK). Fluoresence microscopy was carried out using a Nikon Eclipse TE200 fluorescent microscope and LUCIA image analysis software (Nikon, UK).

Densitometric analysis and statistical analysis

Densitometric analyses were performed using a BioRad GS-710 calibrated imaging densitometer and Quantity One analysis software (Bio Rad, USA). Statistical analyses were performed using one-way analysis of variance and Bonferonni *t*-test. Values of $P<0.05$ were considered significant.

Results

Isolation and characterisation of two splice variants of rat E2F-6

To isolate the rat E2F-6 gene, we designed a set of primers (MM3 and MM4) targeted against highly conserved regions of the ORFs of the mouse and human E2F-6 sequences. RT-PCR using cDNA synthesised from E18 rat ventricular tissue RNA and these primers amplified three distinct PCR products, a 570 bp product (Product 3) that corresponded to the partial sequence of rat E2F-6 as verified by sequencing (Genebank accession number AY211263), a 636 bp product (Product 1) and an approximately 620 bp product (Product 2) (Figure 1A). Since all three products could be amplified from DNase-treated RNA (Figure 1A) and were not amplified from rat liver genomic DNA (data not shown), we concluded that none of these RT-PCR products originated as a result of genomic DNA contamination. We speculated that these products might arise as a result of alternative splicing of the rat E2F-6 gene. Nucleotide sequencing of Product 1 revealed an identical sequence to the rat E2F-6 sequence (Product 3) but with an additional 66-nucleotide insertion at its amino terminus (Genebank accession number: AY211264). Our results are consistent with those reported while this study was in progress, showing that alternative splicing of the mouse E2F-6 gene can give rise to an E2F-6b splice variant containing a 66-nucleotide insert (Kherrouche et al 2001). Thus, our results suggest that Product 1 is the rat version of the E2F-6b splice variant.

Interestingly, the identity of the intermediate-sized PCR product (Product 2) remained elusive. Attempts to clone this PCR product were unsuccessful, with resultant sequence data being identical to either rat E2F-6 or E2F-6b (data not shown). We hypothesized that this product might represent a pseudo-product that was formed as a result of annealing of one DNA strand of E2F-6 with another strand of E2F-6b, giving rise to a double-stranded DNA with an intermediate size (Figure 1B). To test this hypothesis, we used S1 nuclease to digest single-stranded DNA, leaving double-stranded DNA intact (Sambrook et al 1989). Indeed, treatment with S1 nuclease led to the loss of the intermediate-sized product leaving RT-PCR products corresponding to E2F-6 and E2F-6b intact (Figure 1C, lanes 4 vs 5). Taken together, our results are consistent with the notion that the intermediate-sized band is not an alternatively spliced variant of E2F-6 but represents a pseudo-product.

To demonstrate the conservation of the predicted rat E2F-6 and E2F-6b amino-acid sequences with mouse and human sequences, an alignment of these amino-acid sequences is shown (Figure 2).

Developmental expressions of E2F-6 and E2F-6b mRNAs and E2F protein in rat cardiomyocytes

The mRNA expression patterns of E2F-6 and E2F-6b during cardiomyocyte development were determined using specific primers for each splice variant. A forward primer (GB92), designed against the 66 bp insert that is unique to E2F-6b, and

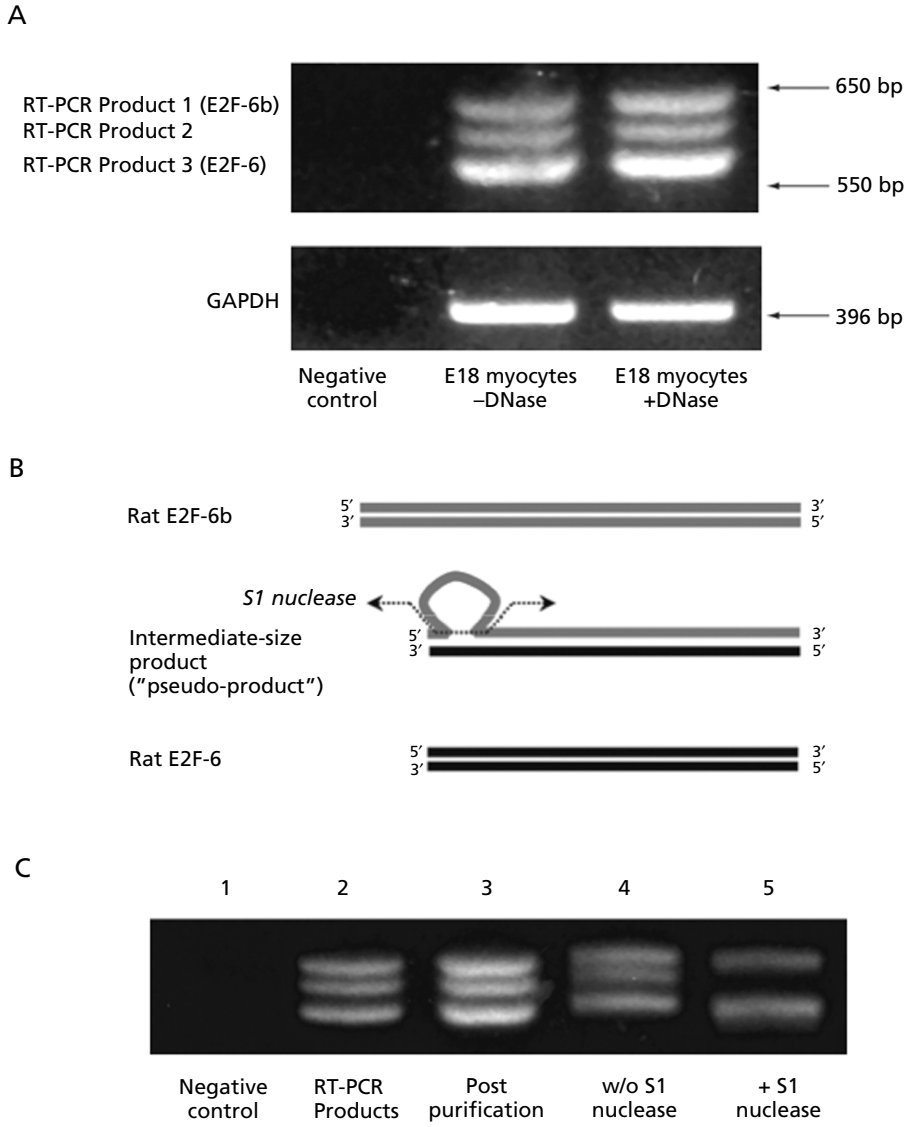

Figure 1 Identification and characterisation of three distinct E2F-6 RT-PCR products amplified from rat cardiomyocyte cDNA. A. Fetal myocyte total RNA was treated with either water (–DNase; lane 2) or 1U of DNase (+DNase; lane 3) before cDNA synthesis and PCR performed using MM3 and MM4 primers. Double-distilled water was used in place of cDNA as a negative control (lane 1). B. Schematic representation demonstrating how intermediate-sized Product 2 could be a pseudo-product comprising one strand of E2F-6b (upper band) and one strand of E2F-6 (lower band) annealing together. Site of action of S1 nuclease enzyme is highlighted (dashed lines). C. RT-PCR products from E18 myocyte cDNA using MM3 and MM4 primers (lane 2) were column purified (lane 3) before incubation of 0.5 μg of DNA without (lane 4) or with (lane 5) 5 U of S1 nuclease enzyme, for 30 min at 37°C.

reverse primer (MM4) were used to amplify E2F-6b. To amplify the E2F-6 isoform, we designed a forward primer (GB91) to the sequences immediately flanking either side of the additional nucleotide sequence that characterised rat E2F-6b. The expression patterns of E2F-6 and E2F-6b mRNAs were determined in E18, P2 and adult rat myocytes. Following normalisation with the house-keeping gene, GAPDH, no significant regulation of either E2F-6 or E2F-6b mRNA was observed during normal development of rat myocytes (Figure 3A). To verify that E2F6 mRNAs were not regulated during normal cardiomyocyte development, we quantitated E2F-6 mRNA expressions using real-time quantitative PCR analysis (Figure 3B). Consistent with our semi-quantitative PCR analysis, quantitative PCR confirmed that E2F-6 mRNAs were not significantly regulated during cardiomyocyte development (Figure 3B).

We next determined the expression pattern of E2F-6 protein during normal cardiac development in E18, P2 and adult myocytes. Using this antibody we detected a band with a molecular weight of approximately 35 kDa (Figure 3C) that corresponded to rat E2F-6 and is in agreement with reports of in-vitro translated mouse EMA that migrates with an apparent Mr of 34 kDa (Morkel et al 1997; Dahme et al 2002). E2F-6 protein expression was down-regulated significantly during cardiomyocyte development (Figure 3D).

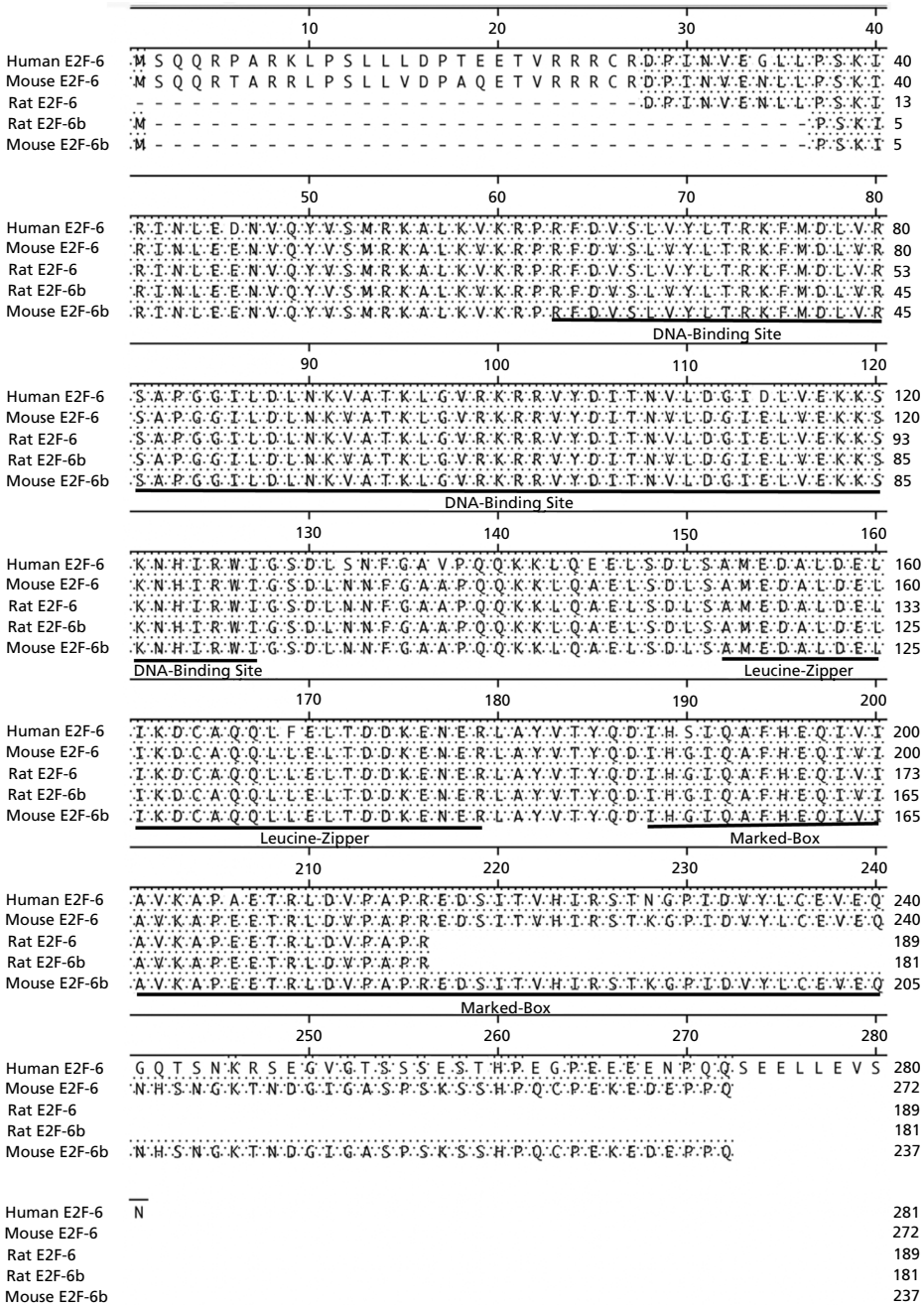

Figure 2 Alignment of human, mouse and partial rat E2F-6 and E2F-6b amino-acid sequences. Alignment of human E2F-6 (accession number AAC31426), mouse E2F-6 (accession number AAL38216), partial rat E2F-6 (accession number AAO59385), partial rat E2F-6b (accession number AAO59386) and mouse E2F-6b (accession number AAL38217) amino-acid sequences. Matching amino-acid sequences have been shaded. The DNA-binding site, leucine-zipper and marked-box consensus sequences have been underlined.

Expression of rat E2F-6 and E2F-6b mRNA and E2F-6 protein during the development of myocyte hypertrophy

The induction of hypertrophy was determined in cultured neonatal myocytes by measuring: the mRNA expressions of the hypertrophic markers, atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP); changes in cell size; and changes in total protein content as described previously (Vara et al 2003). Using semi-quantitative RT-PCR, we demonstrated that no significant change in the expressions of E2F-6 or E2F-6b mRNAs occurred following treatment of cells for 24 h with hypertrophic agonists, 20% FCS, 100 μM PE or 10 nM ET-1 (Figure 4A). Results from our semi-quantitative RT-PCR analysis were verified using quantitative PCR (Figure 4B). Since our developmental expression studies showed that E2F-6 is regulated at the protein rather than mRNA level, we determined the expression pattern of E2F-6

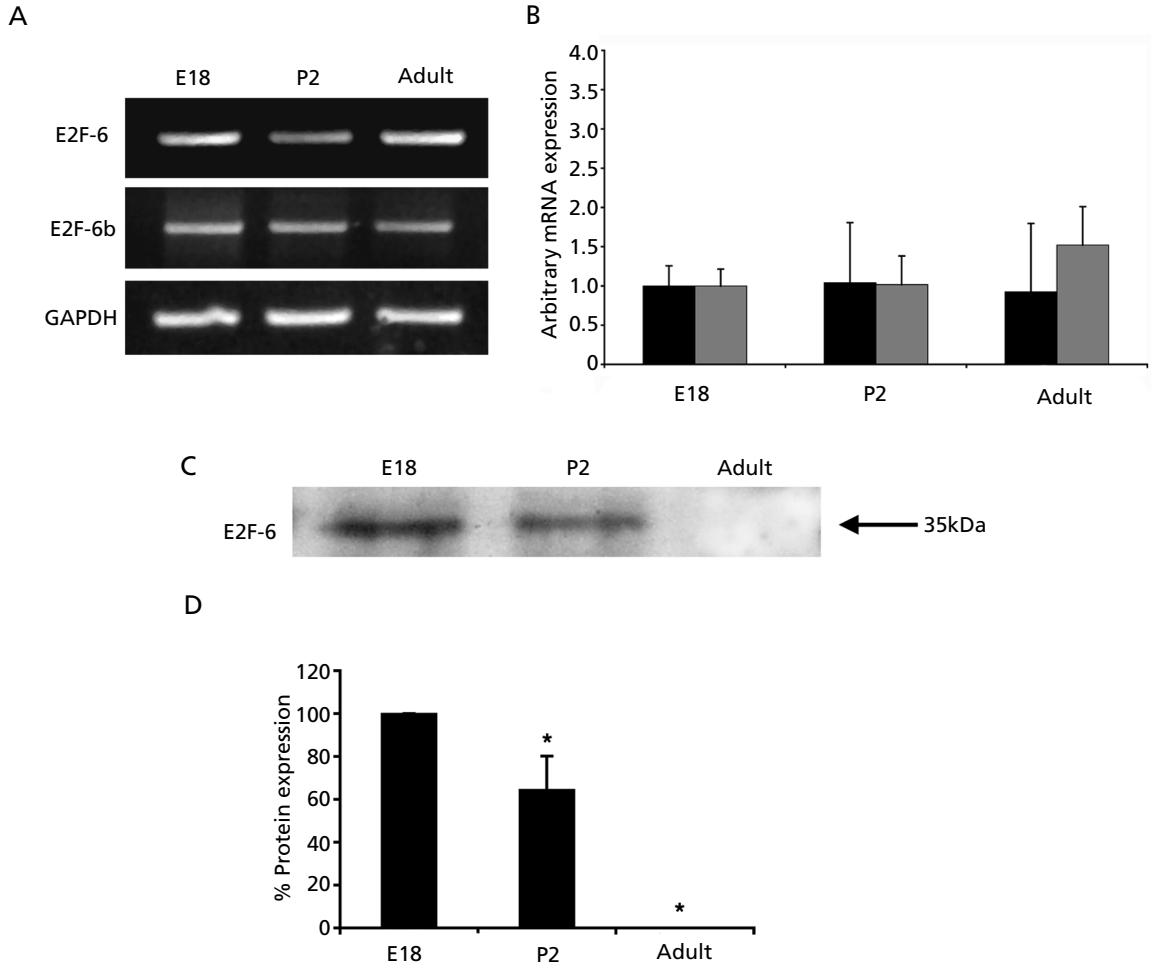

Figure 3 E2F-6 protein expression, but not E2F-6 and E2F-6b mRNA expression, is regulated during cardiomyocyte development. A. Semi-quantitative RT-PCR analysis was performed on fetal (E18), neonatal (P2) and adult rat ventricular myocyte total RNA. Expressions of E2F-6 and E2F-6b were normalised against the house-keeping gene, GAPDH. B. Expressions of E2F-6 mRNAs were measured using quantitative PCR and normalised to GAPDH mRNA expression. Results from 3 or 4 independent myocyte preparations are shown. C. Representative immunoblot to show E2F-6 protein expression in myocytes isolated from fetal (E18), neonatal (P2) and adult rat ventricles. D. Protein expression in developing rat cardiomyocytes is shown graphically. Expression data (mean ± s.d.) were obtained from at least three separate experiments. $*P < 0.05$ vs E18.

protein during the development of hypertrophy. Our results showed a substantial increase in the expression of E2F-6 protein during the development of hypertrophy with all hypertrophic agonists examined (Figure 4C); this up-regulation was more pronounced with 20% FCS (216±15%) than with 100 μM PE (166±25%) or 10 nM ET-1 (152±24%) (Figure 4D).

Transfection of rat neonatal myocytes with human E2F-6

Since E2F-6 protein levels decreased dramatically during myocyte development (Figure 3) and correlated with the loss in proliferative capacity of cardiomyocytes (Poolman & Brooks 1998), we investigated whether E2F-6 could modulate proliferation in these cells. Cultured neonatal rat myocytes were transfected with pAG-IRES2-EGFP or human E2F-6 cloned into pAG-IRES2-EGFP vector in the anti-sense orientation. Following transfection, myocytes were cultured for 48 h with 5% FCS myocyte media post-transfection. Each experiment was performed in triplicate and the results are a combination of three separate experiments.

Western blot analysis demonstrated that the expression of E2F-6 protein was down-regulated following transfection of myocytes with the E2F-6 anti-sense construct (Figure 5A). Furthermore, transfection of neonatal cardiomyocytes with E2F-6 anti-sense vector resulted in a significant reduction in the number of sarcomeric tropomyosin-positive cardiomyocytes (58±7%) 48 h post-transfection, compared with control transfections with vector alone (112±22%, n=3; $P < 0.05$) (Figure 5B). This reduction was due to a reduction in the number of cardiomyocytes present and not inhibition of cardiomyocyte proliferation, since we observed no significant change in the number of myocytes present at the time of transfection compared with those present following transfection with vector alone (100±8%, T=0 h vs 112±22%, T=48 hours) (Figure 5B). To determine the effect of E2F-6 over-

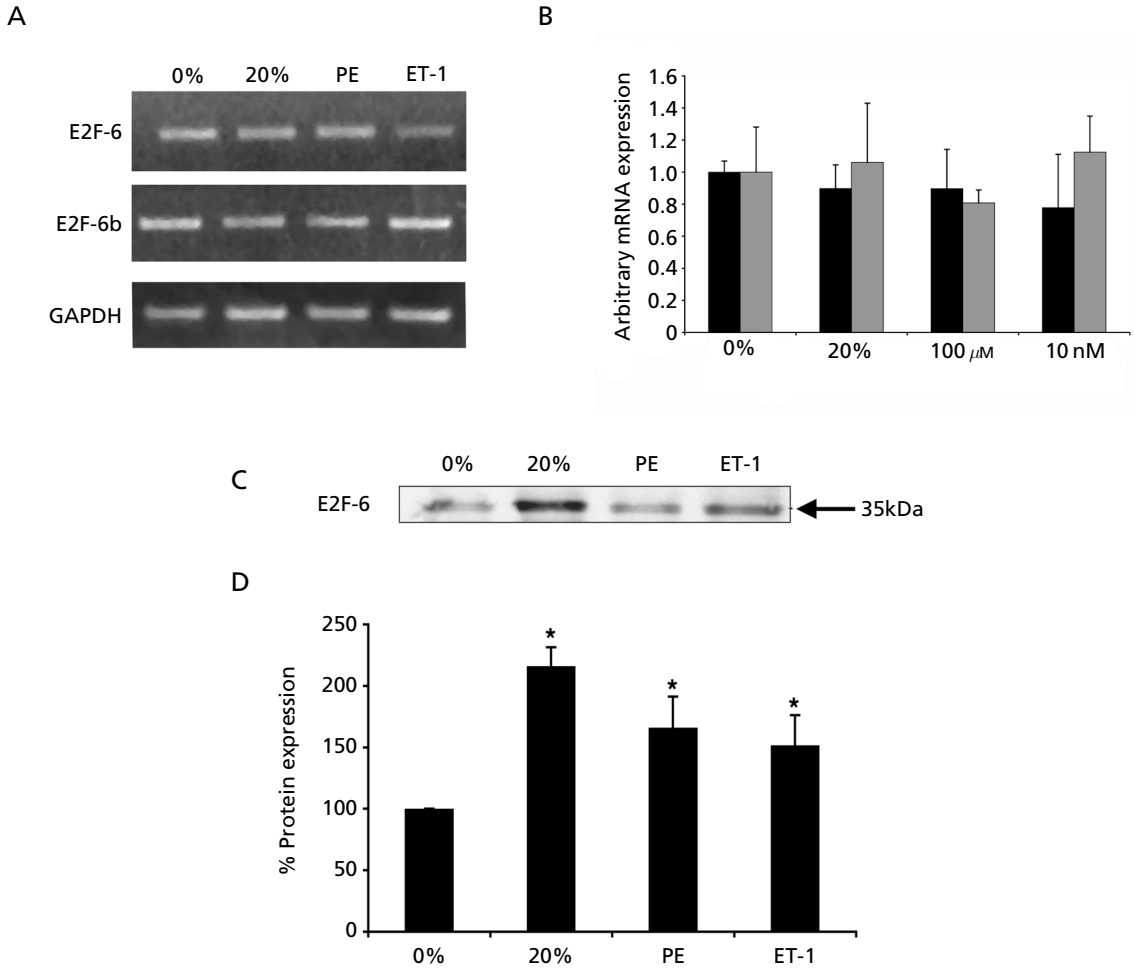

Figure 4 E2F-6 protein expression, but not mRNA expression, is regulated during cardiomyocyte hypertrophy. A. Semi-quantitative RT-PCR analysis was performed on total RNA extracted from myocyte cultures treated with 0% FCS, 20% FCS, 100 μM PE or 10 nM ET-1 for 24 h. Expressions of E2F-6 and E2F-6b were normalised against the house-keeping gene, GAPDH. B. Quantitative PCR analysis was performed to verify semi-quantitative PCR data. Results from 3–5 independent in-vitro hypertrophy experiments are shown. C. A representative Western blot shows differential regulation of E2F-6 protein expression during the induction of hypertrophy in rat myocytes 24 h after treatment with 20% serum, 100 μM PE or 10 nM ET-1. D. Protein expression in hypertrophic rat cardiomyocytes is shown graphically. Expression data (mean ± s.d.) were obtained from at least three separate experiments. $P < 0.05$ vs 0% FCS.

expression, we also examined the effect that transfection of an E2F-6 sense construct had on neonatal cardiomyocyte proliferation. Although increased E2F-6 protein expression was observed in neonatal cardiomyocytes transfected with the E2F-6 sense construct (Figure 5A), no significant increase in myocyte cell number was observed compared with controls (Figure 5B). Taken together, these results suggest that E2F-6 expression might be important for neonatal myocyte viability, not proliferation, and that inhibition of E2F-6 expression leads to myocyte death.

Discussion

In this study, we have isolated, sequenced and characterised E2F-6, along with a splice variant named E2F-6b, for the first time in rat cardiomyocytes. Furthermore, the regulation of E2F-6 transcripts and protein has not previously been studied during cardiomyocyte development nor during myocyte hypertrophy. Three distinct products were amplified by RT-PCR and shown not to be the result of amplification of contaminating genomic DNA. Purification, cloning and subsequent sequencing of the smallest product (Product 3) demonstrated that it comprised the partial nucleotide sequence of rat E2F-6 that was 95% and 85% identical to the published mouse and human E2F-6 sequences, respectively. The nucleotide sequence of the largest product (Product 1) was identical to E2F-6, except for an additional 66-nucleotide insert at the 5′ end of the sequence. In support of this observation, Kherrouche et al (2001) reported alternative splicing of the mouse E2F-6 gene leading to the expression of an E2F-6b splice variant containing a 66-nucleotide insert at the amino

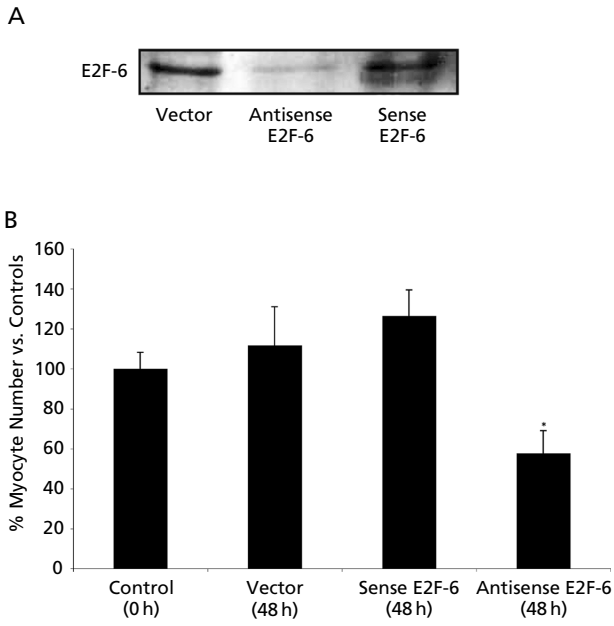

Figure 5 Transfection of an anti-sense human E2F-6 construct, and not a sense construct, in rat cardiomyocytes alters myocyte cell number. A. Western analysis demonstrates E2F-6 protein expression in cultures transfected with sense or anti-sense E2F-6 constructs compared with cultures transfected with vector alone. B. Immunocytochemical analysis was performed to quantitate the effect that the sense and antisense E2F-6 constructs had upon myocyte cell numbers. The number of tropomyosin-positive cells in transfected cultures 48 h post-transfection (t = 48 h) is presented graphically as means ± s.d. (n = 3). The number of myocytes present at the time of transfection (t = 0 h) also is shown. Cell numbers are represented as percentage values where 100% represents total cell number at time of transfection. $*P < 0.05$ vs controls.

terminus. Thus, Product 1 represents the rat version of E2F-6b, with 64 out of the 66 nucleotides within this insert being identical between rat and mouse sequences (95% homology).

An ontogeny study showed that E2F-6 and E2F-6b mRNAs are expressed in fetal (E18), neonatal (P2) and adult rat myocytes with no significant change in expression being observed throughout this period. This finding is consistent with previous reports demonstrating expression of E2F-6 mRNA in adult human heart tissue (Cartwright et al 1998; Trimarchi et al 1998; Dahme et al 2002) and in whole fetal mouse tissues (Kherrouche et al 2001). Furthermore, Dahme et al (2002) recently have shown that E2F-6b mRNA is expressed ubiquitously alongside E2F-6 in all primary mouse tissues examined and that both transcripts are highly expressed, in equal abundance, in adult mouse heart tissue. Our findings in isolated adult rat cardiomyocytes are in agreement with this report. Our results also confirm the expected strong homology between the rat and mouse E2F-6 gene sequences such that, similar to our observations, only 2 mRNA species have been detected in mouse (Kherrouche et al 2001). In contrast, it has been reported that the human E2F-6 gene gives rise to at least 6 different alternatively spliced mRNA species (Kherrouche et al 2004). However, no variations have been reported in the expressions of the E2F-6 isoforms during the cell cycle in various cell lines, suggesting they all probably serve a common function in the cell (Kherrouche et al 2004).

Although E2F-6 mRNA levels did not alter during normal rat myocyte development, E2F-6 protein was down-regulated significantly, suggesting that this molecule is regulated at the post-transcriptional level. Our observations are inconsistent with one report suggesting that E2F-6 has a role in repressing genes in the G_0 phase of the cell cycle (Ogawa et al 2002). Since adult myocytes are arrested in G_0/G_1 (~85%) and G_2/M (~15%) phases of the cell cycle (Poolman et al 1998), we might have expected an up-regulation of E2F-6 protein in adult myocytes that had exited the cycle. In contrast, we did not detect any E2F-6 protein in adult rat myocytes, suggesting that E2F-6 protein is unlikely to play a significant role in the developmental exit of adult rat myocytes from the cell cycle.

We next examined the expression of E2F-6 and E2F-6b transcripts during the development of myocyte hypertrophy using an established in-vitro model (Vara et al 2003). Semi-quantitative and quantitative RT-PCR showed no significant changes in the expressions of either E2F-6 or E2F-6b transcripts following the induction of hypertrophy by any of the hypertrophic agonists used, whereas all hypertrophic agonists led to significant increase in the expression of the E2F-6 protein when compared with cells maintained in 0% FCS. Interestingly, the up-regulation of E2F-6 protein in hypertrophy closely matches the progression of myocytes from G_1 to the S-phase of the cell cycle during this process (Li et al 1998). Other investigators have reported that transcription of E2F-6 (Kherrouche et al 2001; Dahme et al 2002) and E2F-6b (Dahme et al 2002) peak during the late G_1/early S phases of the cell cycle in mouse embryonic fibroblasts (MEFs). Although expressions of E2F-6 and E2F-6b in MEFs appear to be transcriptionally regulated (Kherrouche et al 2001; Dahme et al 2002), in contrast to the post-transcriptional regulation that we observed in rat myocytes, an up-regulation in E2F-6 expression following progression to S phase during the induction of hypertrophy is consistent with the reported maximal expression of this molecule in early S phase in MEFs (Kherrouche et al 2001; Dahme et al 2002).

Ogawa et al (2002) demonstrated that E2F-6 is a component of a larger complex, which exerts transcriptional silencing of genes in the G_0 phase of the cell cycle by recruitment of mammalian PcG to the promoter region. However, their findings are not consistent with the maximal expression level of E2F-6 that we have observed during development of myocyte hypertrophy where there is a transition from the G_1 to S phase of the cell cycle as reported in our study (Li et al 1998) and observations reported by others (Kherrouche et al 2001; Dahme et al 2002). Furthermore, observations by other investigators challenge the notion that the major function of E2F-6 is gene silencing in the G_0 phase of cell cycle, since microinjection of E2F-6 into an asynchronous population of NIH3T3 had no effect on cell cycle profile (Gaubatz et al 1998); also, it remains ambiguous as to why, if E2F-6 is responsible for long-term silencing in G_0, such a complex is recovered from HeLa cells that are unable to enter quiescence (Ogawa et al 2002). On the other hand, it has been demonstrated that ectopic expression of E2F-6 results in accumulation of cells in S-phase, suggesting that E2F-6 may also play a role in

delaying exit of cells out of S-phase (Cartwright et al 1998). In addition, it has recently been demonstrated that E2F-6 associates with the G_1/S activated target genes during S-phase (Giangrande et al 2004). Altogether, these reports are consistent with our findings regarding the expression profile of E2F-6 protein, and suggest E2F-6 likely plays an additional role to gene silencing in G_0 and modulates S phase and the G_1/S transition in cardiomyocytes (this report) and in other cell lines (Cartwright et al 1998; Kherrouche et al 2001; Dahme et al 2002).

To investigate a possible functional role for E2F-6 in myocytes, we transiently transfected cultured rat neonatal myocytes with sense or antisense human E2F-6 constructs. Our studies demonstrated that transfection of cardiomyocytes with the E2F-6 sense construct did not have a significant effect in myocyte number compared with vector alone. This lack of effect suggests that E2F-6 protein might not play a significant role in regulating myocyte proliferation. In support of our observations, it previously has been reported that ectopic expression of E2F-6 in asynchronous Saos-2 or NIH3T3 cell lines does not effect the cell cycle profile or the proliferative capacity of these cells (Gaubatz et al 1998). Interestingly, transfection of neonatal cardiomyocytes with the antisense E2F-6 construct specifically decreased myocyte number when compared with transfection with vector alone. This decrease in myocyte number is most likely due to death of myocytes specifically transfected with the E2F-6 antisense construct via necrosis or apoptosis. In accordance with this hypothesis, Oberley et al (2003) have reported that E2F-6 exerts transcriptional repression of genes that regulate tumour suppression and chromatin modification by a mechanism independent of methylation of the lysine 9 residue in histone H3. These investigators found that depletion of E2F-6 using RNAi resulted in recruitment of E2F-1 to the target promoter. It has also been demonstrated that E2F-6 represses G_1/S phase genes during S phase exit and that depletion of E2F-6 can be compensated for by E2F-4 in E2F-6$^{-/-}$ MEFs (Giangrande et al 2004). Considering reports that inappropriate expression of E2F-1 in cardiomyocytes leads to apoptosis (von Harsdorf et al 1999) and that over-expression of E2F-6 reduces apoptosis by repressing promoters of apoptosis-inducing genes, such as E2F-1 (Furukawa et al 2000), an interesting possibility exists that might explain our observations. Thus, depletion of E2F-6 in neonatal cardiomyocytes might result in the de-repression of a subset of apoptotic genes, which could occur via displacement of E2F-6 with E2F-1 from the promoter region of such genes and hence transcriptional activation of apoptotic genes resulting in myocyte death. Although we do not have experimental evidence to support this hypothesis, it is consistent with the relatively low expression levels of E2F-4 protein (that might have otherwise compensated for the depletion of E2F-6 function) and a relatively high expression of E2F-1 protein that might contribute to induction of apoptosis in neonatal myocytes (Vara et al 2003). This possibility warrants future investigation. Furthermore, the fact that E2F-6 levels affect cell growth, and that expression of this molecule mirrors very closely the proliferative capacity of cardiomyocytes, suggests E2F-6 might be a useful therapeutic target for encouraging the regeneration of new myocardial tissue following MI.

Conclusions

Here, we report for the first time the isolation and characterisation of rat E2F-6, along with a splice variant named E2F-6b. In summary, we have demonstrated that E2F-6 and E2F-6b mRNAs are expressed in rat cardiomyocytes and have shown that E2F-6 protein is down-regulated developmentally, and up-regulated during the development of cardiomyocyte hypertrophy. The expression pattern of E2F-6 protein during this developmental withdrawal of myocytes from the cell cycle and during the development of myocyte hypertrophy when myocytes re-enter cell cycle suggests a potential role for E2F-6 in the G_1/S transition or S-phase (or both) of the cell cycle in rat cardiomyocytes. Furthermore, we have demonstrated that E2F-6 is important for viability of neonatal myocytes and that depletion of this protein at this stage of development leads to myocyte death, thereby suggesting E2F-6 as a potential target for myocardial regeneration.

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