

Gata4 and Sp1 regulate expression of the erythropoietin receptor in cardiomyocytes

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Abstract

Experimental studies indicate significant cardioprotective effects of recombinant erythropoietin (Epo) by binding to the Epo receptor (EpoR) and by inducing various molecular mechanisms, including activation of Gata4, a transcription factor that induces anti-apoptotic genes. However, specific molecular mechanisms of *EpoR* regulation in cardiomyocytes are unknown. We identified a 774 bp regulatory domain in the *EpoR* 5' flanking region by reporter gene assays in murine HL-1 cardiomyocytes. The binding sites for Gata and Sp transcription factors both significantly contributed to *EpoR* promoter activity. DNA-binding studies (EMSA and ChIP assays) identified Gata4 and Sp1 as *EpoR* promoter-binding proteins in HL1 cardiomyocytes. Although Sp1 alone stimulates *EpoR* only slightly, forced expression of *Gata4* significantly induced *EpoR* mRNA expression. In addition, knockdown of Gata4 (but also of Sp1) resulted in a significant decrease of *EpoR* transcript levels in HL-1 cardiomyocytes. Cumulative *in vitro* data suggest that function of the Sp1 site is essential for the Gata4-mediated transcription. *In vivo*, analysis of transgenic mice expressing an inducible small-hairpin RNA against *Gata4* confirmed suppression of *EpoR* expression in the heart. Treating mice with high-dose doxorubicin not only resulted in Gata4 protein depletion, but also down-regulated *EpoR*, followed by up-regulation of *EpoR* transcripts when Gata4 levels recovered. In conclusion, we identified Gata4 as novel regulator of *EpoR* transcription in cardiomyocytes. In models of cardiac injury, down-regulation of Gata4 or Sp1 may limit the accessibility of the EpoR for binding of erythropoiesis-stimulating agents (ESA). Thereby our data underline the essential role of Gata4 in mediating cardioprotective effects.

Keywords: cardioprotection • erythropoietin • erythropoietin receptor • GATA4 • transcription

Introduction

Animal studies indicate that recombinant erythropoietin (rEpo) or other erythropoiesis-stimulating agents (ESA) exert cardioprotective effects in ischaemia-reperfusion injury and also in non-ischaemic cardiac dysfunction [1, 2]. The *in vivo* effects of rEpo include enhanced cardiac functional recovery, better left ventricular contractility, decreased infarct size, suppressed myocardial inflammation, reduced apoptosis and decreased remodelling. Although the underlying molecular mechanisms are

not entirely clear, recent data indicate that rEpo restores protein levels of the cardiac transcription factor Gata4 in cardiomyocytes treated with doxorubicin [3], a frequently used anthracycline causing cardiomyopathy in cancer treatment, or after cardiac ischaemic-reperfusion injury [4]. Gata4 regulates genes that are relevant for proper cardiomyocyte integrity and function, such as α -myosin heavy chain (α -Mhc), β -Mhc, troponin, atrial natriuretic factor (*Anf*) and bone morphogenetic protein-4 (*Bmp4*) [5, 6]. Because Gata4 exhibits direct anti-apoptotic function by regulating genes of the *bcl-xL* family and other factors [4, 7], rEpo appears to be an attractive pharmaceutical substance to prevent or even to treat cardiomyopathy *via* Gata4 restoration [8, 9]. However, rEpo or its derivatives have not been tested clinically for anthracycline-induced cardiomyopathy, and the first clinical data on rEpo for the protection against ischaemia-reperfusion heart injury are rather disappointing if compared to experimental data [10]. Because Epo's actions require binding to

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the extracellular domain of the Epo receptor (EpoR), the question on the regulation of the *EpoR* gene in cardiomyocytes is of particular interest.

The first evidence for an essential role of Epo and its receptor in the heart resulted from the analysis of transgenic mice with homozygous deletion of the *Epo* (*Epo*^{-/-}) or the *EpoR* (*EpoR*^{-/-}) genes [11, 12]. Both *Epo*^{-/-} and *EpoR*^{-/-} embryos suffer from ventricular hypoplasia, epicardium detachment and vascular abnormalities [11]. In the murine heart, *EpoR* is expressed in a temporal and cell type-specific manner. From mid-gestation onwards, *EpoR* expression has been detected in foetal, neonatal and adult cardiomyocytes [13–16]. Organ cultures or primary cell cultures from embryonic heart showed that rEpo acts as mild mitogen for cardiomyocytes [11]. In *EpoR*^{-/-} mice that have been rescued from the lethal haematopoietic defect, the endogenous EpoR system is relevant for protection against pressure-overload induced cardiac dysfunction and for protection against myocardial ischemia/reperfusion injury [17, 18]. Although low *Epo* transcript levels have been detected in the murine embryonic and human foetal heart [11, 14], significant *Epo* expression in cardiomyocytes was not observed under normoxia or hypoxia [15]. This supports the hypothesis that the EpoR has the major implication in mediating the effects of endogenous Epo in cardiac morphogenesis and of rEpo for cardioprotection, even if the EpoR number is low under normal conditions.

The regulatory mechanisms of *EpoR* expression in cardiomyocytes in health and disease are unknown yet. In haematopoietic cells, transcriptional regulation of *EpoR* gene is controlled by *cis*-acting elements [19]. Earlier studies showed that the transcription factors Gata1, Sp1 and Wt1 activate *EpoR* gene expression in haematopoietic cells by binding to a 452 bp minimal *EpoR* promoter element [20–23]. However, Gata1 expression is restricted to haematopoietic progenitor cells and Sertoli cells [24]. The analysis of *EpoR* gene expression in the heart of *Wt1*-deficient embryos revealed that the transcriptionally active *Wt1* protein was not involved in cardiac *EpoR* expression [23]. Because the transcriptionally active *Wt1* protein is expressed exclusively in the epicardium [25], however, further experiments may be required to test whether *Wt1* affects *EpoR* expression in cardiogenic progenitors of the epicardium that are involved in cardiac regeneration.

To improve the translation of experimental work on cardioprotection by rEpo or ESA into future clinical strategies, we aimed to elucidate the regulation of *EpoR* gene expression in cardiomyocytes. Herein, we provide the first evidence that Gata4 specifically activates *EpoR* expression in cardiomyocytes.

Material and methods

Cell culture

The murine cardiomyocyte cell line HL-1 was cultured as described [26].

Animal experiments

The investigations conform to 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).

Doxorubicin-induced cardiomyopathy

Cardiomyopathy was induced by a single intraperitoneal injection of 15 mg/kg doxorubicin-HCl (Merck, Darmstadt, Germany) in 10-weeks-old male C57BL/6J mice ($n = 9$). In sham-treated mice ($n = 6$), the equal volume of saline was intraperitoneally injected. Mice were sacrificed after 24 hrs ($n = 6$) or 5 days ($n = 3$), respectively, and hearts were isolated for RNA and protein preparation (IRB Protocol LaGeSo Berlin G0224/06).

Transgenic mice expressing short hairpin RNA (shRNA) against Gata4

The transgenic mouse line (H1:*G4/TetR*), exhibiting an inducible shRNA construct against *Gata4*, has been recently developed by us [27]. To induce expression of *Gata4* shRNA via the *TetR* system, mice ($n = 4$) were fed with 20 mg doxycycline per ml drinking water. The control group ($n = 4$) was fed with doxycycline at the same concentration. After 38 days mice were sacrificed, and hearts were taken for analysis (IRB Protocol D669, University of Florida, USA).

RT-PCR

For RNA preparation, tissue specimens were homogenized in TRIzol® (Invitrogen, Karlsruhe, Germany) reagent. RNA was isolated according to the manufacturer's protocol. Subsequently, cDNA was synthesized and transcripts of interest were amplified by using specific primers (Table 1).

Quantitative real-time PCR (Q-PCR)

For Q-PCR, 5'-FAM-labelled probes of murine *Gata4*, *EpoR* (TaqMan Gene Expression Assays; Mm00484689, Mm00833882) and β -actin (4352933E) from Applied Biosystems (Foster City, CA, USA) were used. PCR reactions were performed as described [28]. The threshold value was calculated using the iCycle iQ Optical System Software, version 3.1. Data were normalized against β -actin.

Plasmids and site-directed mutagenesis

Firefly luciferase reporter gene constructs were generated by cloning various EpoR promoter fragments into the pGL2-basic vector (Promega, Mannheim, Germany). For preliminary experiments, a 5 kb fragment of the 5'-flanking region of the murine *EpoR* gene (NCBI accession no. NC_000075) was cloned, spanning the sequence from nucleotide -4900 to +100 relative to the transcription start site. This fragment was shortened to generate two additional constructs containing the either an 1842 bp fragment (-1742 to +100) and/or a 774 bp fragment (-674 to +100; Fig. S1). The latter construct, named full-length *mEpoRpro*, was used for detailed analysis. Potential

Table 1 Sequences of primers for RT-PCR analysis in murine tissue specimens and HL-1 cells

| Gene | Accession no. | Forward primer | Reverse primer | Product size (bp) |
|----------------|---------------|--------------------------|--------------------------|-------------------|
| <i>β-actin</i> | NM_007393.2 | actgctctggctcctagcac | acatctgtggaaggtggac | 115 |
| <i>EpoR</i> | NM_010149 | agccccgctcactactgacc | gtcctgggcatgctcactgc | 386 |
| <i>Epo</i> | NM_007942 | ctgggagctcagaaggaattgatg | ctggagtgtccatgggacagactg | 340 |
| <i>Gata1</i> | NM_008089 | gagtccagacctcctgacgc | cagtgtagtagccgttgctc | 220 |
| <i>Gata4</i> | NM_008092 | gatgggacgggacactacctg | acctgtggcgtcttagattt | 288 |
| <i>Gata6</i> | NM_010258 | gcacgcttccctactcgc | cgagttaggtcgggtgatgg | 222 |
| <i>Fog2</i> | NM_011766 | gcaagctcagacacagagc | ccagctcggacatcttcgc | 276 |
| <i>Sp1</i> | NM_013672 | ggatgaagtgcacagctgtgg | gaagagatgatctgccagcc | 318 |
| <i>Wt1</i> | NM_144783 | agagccagcctaccatccgc | ggctcctgtgcaactgtca | 221 |
| <i>α-Mhc</i> | NM_010856.3 | ttagctggaagatcaccgg | aagtcagagaaggaacgcctaga | 783 |

All sequences are shown in 5'–3' direction.

binding sites for transcription factors were mutated within the pGL2-mEpoRpro construct by using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). For details and primer sequences see 'Supporting information'.

The murine *Gata4* cDNA (NM_008092; bp 608–1933) was cloned into the EcoRI site of pcDNA3.1(+) vector (Invitrogen). A XhoI/EcoRI fragment containing the open reading frame of *Sp1* cDNA (NM_013672; bp 101–2446) was cloned into the corresponding site of pcDNA3.1(–). The pCMVTag3B/*Gata-6* vector was kindly provided by Edward Morrisey [29]. pBS/U6-shScram and pBS/U6-shSp1–2 vectors were kindly provided by Grace Gill [30].

Reporter gene assays

Four micrograms of each reporter gene construct and 20 ng of the *Renilla* expression plasmid phRL-TK were transiently co-transfected into HL-1 cells performed with Lipofectamine™ 2000 (Invitrogen). After 24 hrs cells were lysed and the luciferase activities were measured (Dual-Luciferase Reporter Assay System, Promega). Relative light units (RLU) of firefly luciferase were normalized against *Renilla* luciferase-activities and protein concentrations. The amount of total protein was determined using Bradford reagent.

Nuclear extracts, oligo-labelling and electrophoretic mobility shift assay (EMSA)

EMSA oligonucleotides of the *EpoR* promoter region (NCBI accession no. NC_000075; –74/–45 GATA 5'-gttcccttggtgctgtagcttatctgtcc-3'; –44/–14 GC-rE 5'-ccagcctgcaagctggccccgcccctgga-3') were end-labelled with ³²P and subsequently incubated with 5 μl of HL-1 nuclear extract. Where indicated, the GATA-4, GATA-6 or Sp1 antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with freshly prepared cardiomyocytes from three male 10-week-old C57/BL6 mice by using the ChIP Assay Kit

(Millipore, Schwalbach/Ts, Germany) according to manufacturer's instructions. See 'Supporting information' for technical details.

Forced expression of transcription factors

For transient overexpression, 4 μg of each plasmid were transfected into HL-1 cells performed with Lipofectamine™ 2000. After 48 hrs, RNA was extracted, and cDNA was synthesized. The effects of overexpression on *EpoR* mRNA abundance were quantified by Q-PCR.

Gata4 and Sp1 knockdown in HL-1 cells

pBS/U6-shSp1 or *Gata4* siRNA duplexes were transfected into HL-1 performed with Lipofectamine™ 2000. Forty-eight hours past transfection, RNA and protein were extracted and analysed for *EpoR* mRNA or Sp1 and Gata4 protein, respectively. See 'Supporting information' for details.

Western blot analyses

Proteins were extracted from HL-1 cells or murine heart were performed as previously described [28]. The following primary antibodies were used: GATA-4, GATA-6, Sp1 (Santa Cruz Biotechnology) and fl-tubulin (Promega). Signals were visualized using Western Blot ECL reagent (Pierce, Rockford, IL, USA).

Statistical analysis

Data are presented as mean plus S.D. ANOVA with Bonferroni test as *post hoc* test calculations and Student's t-test were performed as indicated to reveal statistical significances. *P*-values < 0.05 were considered statistically significant.

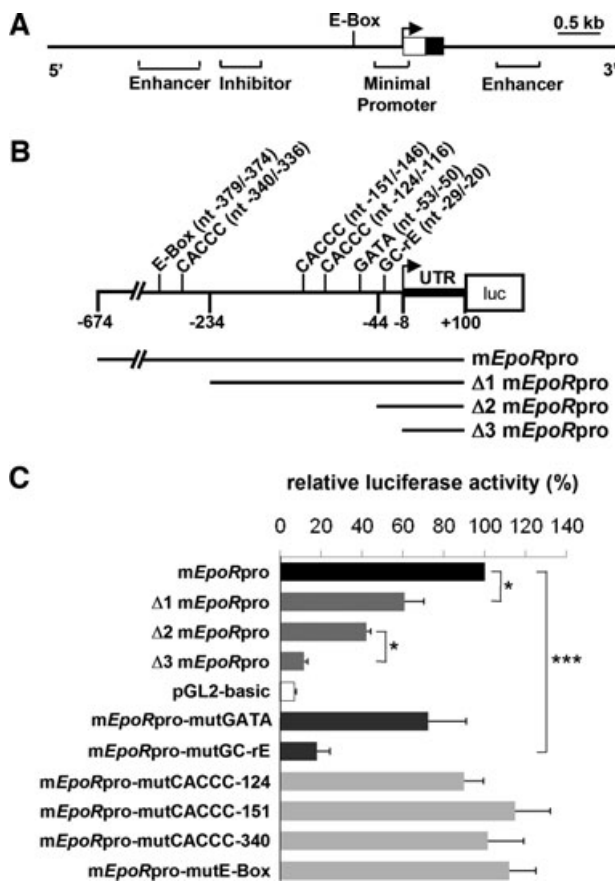


Fig. 1 (A) Diagram of the murine *EpoR* gene locus highlighting the upstream promoter region. The open box displays the 5'-untranslated region and the filled box represents exon 1. Known enhancer and inhibitory elements are indicated [19, 47]. The transcription start site is marked by an arrow. (B) Location of putative *cis*-regulatory elements in the analysed 774 bp *EpoR* promoter element and constructs used for reporter gene assays. Numbers indicate the position of the nucleotides (nt) relative to the transcription start site and refer to various lengths of the reporter gene constructs. *mEpoRpro* represents the 774 bp murine *EpoR* promoter cloned into the luciferase reporter plasmid pGL2. The truncated promoter regions $\Delta 1mEpoRpro$, $\Delta 2mEpoRpro$ and $\Delta 3mEpoRpro$ are illustrated. Specific DNA-binding motifs for transcription factors are indicated with the nucleotide position numbers: one E-box, three CACCC motifs, the GATA element and one GC-rich element, which represents putative binding sites for Sp1 and Wt1. (C) Reporter gene activities after transfection of wild-type or mutant promoter constructs into HL-1 cells. The full-length *EpoR* promoter construct (*mEpoRpro*) and the three truncated forms were compared to the pGL2-basic reporter construct alone. The specific implication of the DNA-binding motifs of interest has been analysed by site-directed mutagenesis of the GATA element, the GC-rich elements, the CACCC motifs and the E-box. Firefly luciferase activities were normalized to the corresponding *Renilla* luciferase values and protein concentrations ($n = 3$ independent experiments; *** $P < 0.001$; * $P < 0.05$).

Results

Regulatory elements upstream of the murine *EpoR* gene

Considering previous studies that identified regulatory domains in the upstream region of the *EpoR* gene, we first analysed a 5 kb fragment, spanning from the nucleotide -4900 to $+100$ relative to the transcription start site of the 5'-flanking region of the murine *EpoR* gene (Fig. S1A). This reporter gene includes the minimal *EpoR* promoter element as well as an enhancer and an inhibitor element that have previously been identified in haematopoietic progenitors, bone marrow stroma cells or fibroblasts [31, 32]. Removing the 5'-flanking enhancer and inhibitor sequence, which are regulated in a lineage- and temporal-specific manner, led to an increase in reporter gene activity. The shortened 774 bp fragment retained an E-box and the minimal haematopoietic *EpoR* promoter (Fig. S1B). In the subsequent experiments, we further dissected the 774 bp fragment of the murine *EpoR* gene (Fig. 1A) [19, 20]. This 774 bp fragment (*mEpoRpro*), spanning from nucleotides (nt) -674 to $+100$ relative to the transcription start site (Fig. 1B), contains various putative transcription factor binding sites, including one E-box, several CACCC-motifs, one GATA site and a proximal GC-rich element (GC-rE). For reporter gene analysis, *mEpoR* promoter constructs were transiently transfected into HL-1 cardiomyocytes. Compared to the full-length *mEpoRpro*, the truncated reporter construct $\Delta 1mEpoRpro$ (nt-234/+100) showed a reduction of activity to $60 \pm 9.7\%$, and a further truncation ($\Delta 2mEpoRpro$, nt-44/+100) reduced reporter gene activity to $42 \pm 2.2\%$. $\Delta 3mEpoRpro$ (nt-8/+100), which contained only the 5'-untranslated region, showed very low activity ($11 \pm 1.9\%$) compared to *mEpoRpro*, and was similar to the empty pGL2 basic vector ($7 \pm 0.8\%$; Fig. 1C). Mutations of the GC-rE resulted in a significantly reduced activity, compared to that of the full-length *mEpoRpro* reporter gene (down to $20 \pm 6.4\%$). Mutation of the GATA-site showed a moderate reduction in luciferase activity (down to $72 \pm 18.6\%$), whereas mutations of the E-box or various CACCC-motifs had no effect (Fig. 1C). The combined data highlight the functional implication of the 774 bp fragment and particularly of its GATA-site and GC-rich elements in mediating *EpoR* promoter activity.

Expression pattern of candidate factors for *EpoR* regulation in the murine heart and HL-1 cardiomyocytes

Next, we analysed the developmental expression profile of *Epo*, *EpoR* and transcription factors that could potentially bind to the *EpoR* promoter as annotated by TRANSFAC analysis or based on

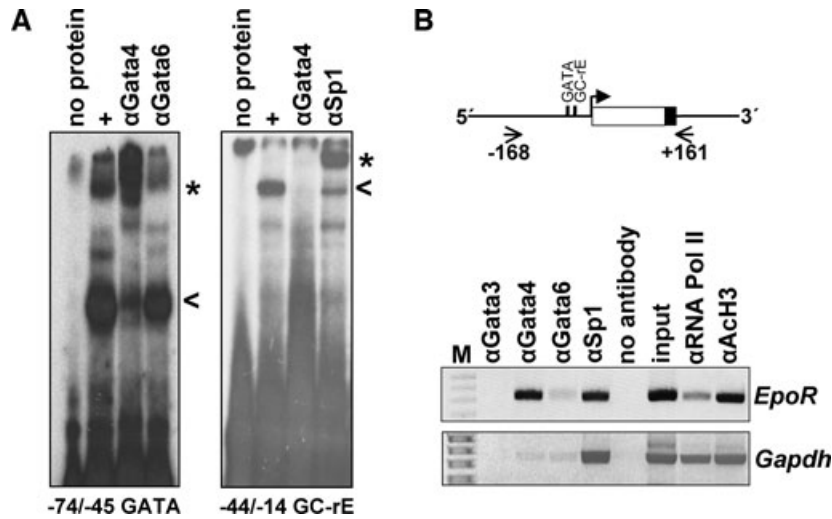


Fig. 2 Analysis of Gata4 and Sp1 binding to the murine *EpoR* promoter. **(A)** Electrophoretic mobility shift assays demonstrate binding of Gata4 (probe nt-74/-45 GATA) and Sp1 (probe nt-44/-14 GC-rE) to DNA elements of the murine *EpoR* promoter. Asterisks indicate specific Gata4 and Sp1-mediated supershifts by antibodies directed against Gata4 (α Gata4), Gata6 (α Gata6) and Sp1 (α Sp1). Of note, incubation with an antibody directed against Gata4 totally abolishes the specific complex generated by binding of Sp1 to the -44/-14 GC-rE. **(B)** Chromatin-immunoprecipitation assays with the *EpoR* minimal promoter were performed in freshly prepared cardiomyocytes (male C57/BL6). Immunoprecipitation with an antibody against Gata3 (α Gata3) served as negative control, whereas that with the antibodies against RNA polymerase II (α RNA Pol II) and against acetylated histon 3 (α ACh3) as positive controls with primers spanning the *EpoR* promoter (nt-168/+161). Amplification of the *Gapdh* promoter served as control. Gel pictures were inverted.

indicate active transcription of the *EpoR* gene. Precipitated DNA was amplified by PCR with primers spanning the *EpoR* promoter (nt-168/+161). Amplification of the *Gapdh* promoter served as control. Gel pictures were inverted.

previous data on *EpoR* regulation in haematopoietic cells [20–23, 32, 33]. Although *Epo* gene expression was silenced in the developing and adult heart, *EpoR* transcripts were detected at all stages (Fig. S2). Heart tissue specimens were negatively tested for *Gata1* mRNA. Similarly, *Wt1* mRNA, which is only expressed at low levels in the epicardium [34], was not detectable. Among factors regulating *EpoR* expression in haematopoietic cells, *Sp1* was expressed throughout development and adulthood. As expected according to the literature [35], transcripts of *Gata4*, *Gata6* and their co-factor friend-of-GATA 2 (*Fog2*) were detected in each specimen. Notably, murine HL-1 cardiomyocytes revealed the same expression profile for the genes expressed in the wild-type murine heart (Fig. S2), suggesting that this cell line is useful and appropriate for later *in vivo* experiments.

***In vitro* and *in vivo* binding of Gata4 and Sp1 to the *EpoR* promoter in cardiomyocytes**

To further explore whether Gata4, Gata6 or Sp1 bind to the *EpoR* promoter, EMSA experiments were carried out with nuclear extracts from HL-1 cells and end-labelled oligonucleotides, which contain regulatory elements of the *EpoR* promoter. By using antibodies, we identified a Gata4, but not Gata6-specific protein/DNA complex, as well as a Sp1-specific protein/DNA complex (Fig. 2A). Of note, the Sp1-specific complex was abolished, if the oligonucleotide that contains only the GC-rich element, but not the GATA-site was incubated with an antibody against Gata4 (Fig. 2A). Thus, Gata4 may be a component of the Sp1-specific complex at the GC-rE. ChIP experiments confirmed that Gata4 as well as Sp1 bind to the *EpoR* promoter in fresh primary cardiomyocytes (Fig. 2B).

Enhanced *EpoR* mRNA expression by forced Gata4 expression

The functional implication of Gata4 and Sp1 binding to the *EpoR* promoter was tested by transient transfection of HL-1 cells. Forty-eight hours after transient Gata4 overexpression a 3-fold stimulation of endogenous *EpoR* expression was found ($P < 0.05$; Fig. 3A), while forced expression of Gata6 had no effect. In contrast to data in haematopoietic cells [22], Sp1 overexpression resulted only in a slight, but not significant increase of *EpoR* mRNA levels in HL-1 cells. Co-transfection of Sp1 with either Gata4 or Gata6 plasmids did also not significantly (further) enhance *EpoR* expression (Fig. 3A). These data clearly support the role of Gata4 as a positive regulator of *EpoR* expression in cardiomyocytes. Unfortunately, the analysis of *EpoR* protein expression could not be performed due to non-specific cross-reactivity of the currently available *EpoR* antibodies [36, 37].

Decreased *EpoR* mRNA expression in Gata4 and Sp1 siRNA experiments

To further study the functional implications of Gata4 and Sp1 on *EpoR* gene regulation in HL-1 cells, we performed knockdown experiments with siRNA. For Gata4 knockdown experiments, scrambled or *Gata4* siRNA oligonucleotides were transfected into HL-1 cells. Forty-eight hours later, protein and RNA were analysed for Gata4 and *EpoR* expression levels. Western blot analysis showed a distinct reduction of Gata4, which is associated with a significant ($P < 0.01$) decrease of *EpoR* mRNA to $63 \pm 6\%$ (Fig. 3B). For Sp1 knockdown, we transfected a short hairpin *Sp1* plasmid or the corresponding scramble control. The Sp1

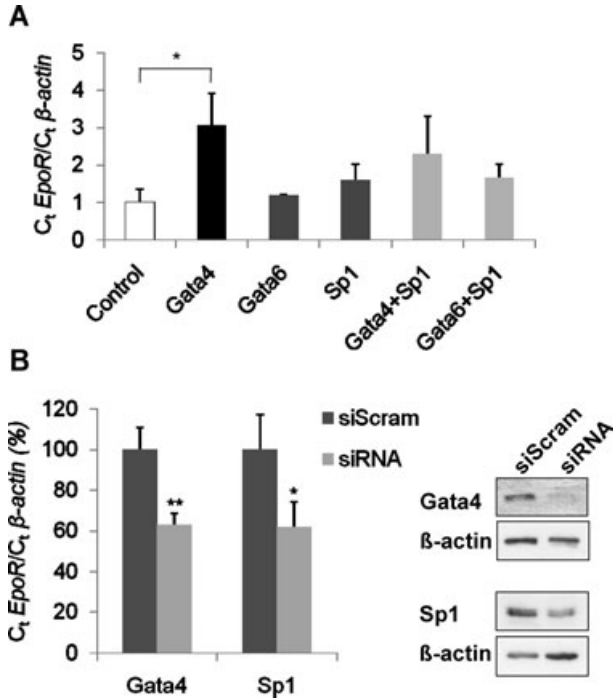


Fig. 3 Influence of Gata4 and Sp1 overexpression or suppression on endogenous *EpoR* expression levels. **(A)** Increase of *EpoR* mRNA by forced expression of *Gata4*. Transcript levels of *EpoR* and β -actin were quantified by Q-PCR 48 hrs after transient transfection of *Gata4*, *Gata6* or *Sp1* (alone or combined) in HL-1 cells. **(B)** Decrease of *EpoR* mRNA by *Gata4* and *Sp1* knockdown. HL-1 cells were transfected with *Gata4* siRNA oligonucleotides and *Sp1* short hairpin vector, respectively. *EpoR* values were normalized to β -actin ($n = 3$ independent experiments; ** $P < 0.01$; * $P < 0.05$). Analysis of *EpoR* protein expression could not be performed due to non-specific cross reactivity of currently available *EpoR* antibodies [36, 37]. *Gata4* and *Sp1* knockdowns were verified by immunoblot.

knockdown was verified by Western blot. Q-PCR analysis showed a clear reduction in *EpoR* mRNA to $55 \pm 13\%$ ($P < 0.05$) upon *Sp1* knockdown (Fig. 3B). Taken together, these experiments underline the implication of *Gata4* and *Sp1* in regulating the *EpoR* gene in HL-1 cardiomyocytes.

Down-regulation of cardiac *EpoR* expression in transgenic mice with inducible RNA interference directed against *Gata4*

To study the effect of *Gata4* knockdown on endogenous *EpoR* expression *in vivo*, we analysed transgenic mice with a stably integrated shRNA against *Gata4* under control of the *TetR* system [27]. To induce the expression of *Gata4* shRNA, transgenic mice were fed with doxycycline over 38 days, because this resulted in the most efficient, significant down-regulation of endogenous *Gata4* mRNA expression to $10 \pm 4.5\%$ of the level in control mice

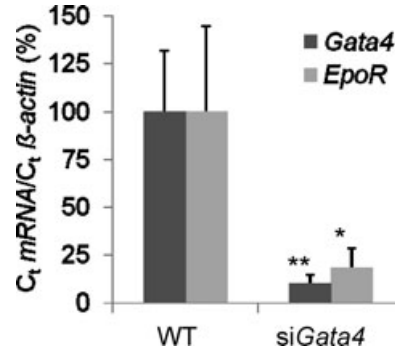


Fig. 4 Inhibition of cardiac *EpoR* mRNA expression in transgenic mice with short hairpin RNA against *Gata4*. Transgenic mice with a stably integrated, doxycycline-inducible *Gata4* shRNA construct and FvB wild-type mice (control) were fed with 20 mg doxycycline per ml drinking water over 38 days. Endogenous *Gata4* and *EpoR* expression levels were quantified by Q-PCR and normalized to β -actin. All samples were measured in duplicates according to the threshold cycle method (** $P < 0.01$; * $P < 0.05$).

($P < 0.01$). In our experiments, reduction of *Gata4* protein levels was associated with the down-regulation of selective *Gata4*-downstream targets, such as the genes encoding *Anf* and *Bmp4*, but not α MHC, and is shown elsewhere [27]. Data from us and others (using conditional cardiac *Gata4* deletion) indicated that under non-stress conditions *Gata4* depletion of up to 80% of normal levels did not result in cardiac failure [6, 27]. However, down-regulation of *Gata4* expression was associated with a significant reduction of *EpoR* mRNA expression to $20 \pm 10\%$ of normal levels (Fig. 4). In addition, *Gata6* levels remained normal in transgenic mice with inducible *Gata4* shRNA [27], confirming our data from DNA-binding analysis and overexpression analysis which indicate that *Gata6* is not involved in cardiac *EpoR* regulation (Figs 2 and 3).

Gata4 depletion in doxorubicin-induced cardiomyopathy is associated with decreased *EpoR* expression

In a more physiological and clinical relevant approach, we studied the role of *Gata4* in regulating cardiac *EpoR* expression in a mouse model of doxorubicin-induced cardiomyopathy. This model is characterized by *Gata4* depletion accompanied with cardiomyocyte atrophy, degeneration and myocardial fibrosis [3]. After a single intraperitoneal injection of 15 mg/kg doxorubicin hydrochloride or the equivalent volume of saline for controls, animals were sacrificed 24 hrs or 5 days later. Cardiac *Gata4*, *Gata6* and *Sp1* levels were analysed by Western blot analysis. *EpoR* levels were quantified by Q-PCR (Fig. 5A). Of note, 24 hrs after single high-dose doxorubicin injection, *Gata4* was depleted, but recovered to almost normal levels after day 5. Coincidentally with *Gata4* depletion, *EpoR* expression

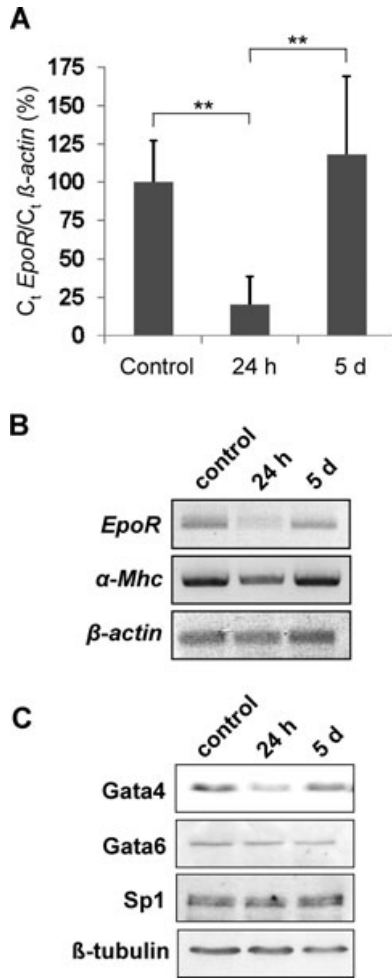


Fig. 5 *EpoR* expression levels in a mouse model of doxorubicin-induced cardiomyopathy following depletion and recovery of Gata4. Cardiomyopathy was induced in 10-week-old male C57BL/6J mice by a single intraperitoneal injection of 15 mg/kg doxorubicin hydrochloride. In sham-treated mice, the equivalent volume of saline was injected. **(A)** Q-PCR analysis of *EpoR* expression, normalized to $\beta\text{-actin}$. All samples were measured in duplicates (** $P < 0.01$). **(B)** Analysis of $\alpha\text{-Mhc}$ expression as Gata4 downstream target served as *bona fide* control for the specificity of the effect. Photographies of agarose gels were inverted. **(C)** Immunoblot of Gata4, Gata6, Sp1 and $\beta\text{-tubulin}$.

significantly decreased (to $19 \pm 18\%$ of normal levels) within the first 24 hrs. After 5 days, *EpoR* mRNA expression recovered in parallel to Gata4 to almost normal levels. Again, Gata6 and Sp1 protein level were unaffected, whereas suppression and later recovery of $\alpha\text{-Mhc}$ mRNA expression served as *bona fide* control for the specific Gata4 effect (Fig. 5B and C). These *in vivo* data also indicate that Gata4 specifically activates *EpoR* expression and may render cardiomyocytes more responsive to rEpo or other ESA.

Discussion

Herein, we identified Gata4 as a novel transcriptional regulator of the *EpoR* gene in cardiomyocytes. This is supported by several findings: Gata4 specifically binds to the *EpoR* promoter *in vitro* (EMSA) and in living cells (ChIP Assay; Fig. 2). Furthermore, mutation of the GATA-motif results in a reduced activity of the *EpoR* promoter (Fig. 1). The combined data from the reporter gene analysis indicate that additional *cis*-regulatory elements are involved in regulating *EpoR* promoter activity (Fig. 1). Our data also confirm that DNA elements in the 5'-flanking region of the murine *EpoR* gene are clearly regulated by both lineage- and temporal-specific mechanisms (Fig. S1) [31]. We also show that Sp1 specifically binds to a GC-rich element within the *EpoR* promoter in cardiomyocytes (Fig. 2). Although forced expression of Sp1 only slightly stimulates endogenous *EpoR* expression, Gata4 significantly enhances *EpoR* mRNA expression (Fig. 3). In an opposite approach, knockdown of Gata4, but also of Sp1 was associated with a significant down-regulation of *EpoR* mRNA expression (Figs 3 and 4). The high expression of Sp1 in HL-1 cardiomyocytes may explain that forced overexpression of Sp1 did not result in a more pronounced induction of *EpoR* mRNA levels. Of note, Gata4 is known to interact physically with Sp1 [38], and EMSA experiments indeed indicate that Gata4 binds to the Sp1-specific complex at the GC-rE in the minimal *EpoR* promoter (Fig. 2A). This suggests that accessibility of Gata4 may be important for Sp1 to activate *EpoR* expression or *vice versa*. In haematopoietic progenitor cells, synergistic effects of Sp1 and Gata1 have previously been proposed for *EpoR* regulation and may also depend on the stage of cellular differentiation [22, 23]. In neuronal cells, Gata2 and Gata3 bind to the same GATA-motif in the *EpoR* promoter, but this does not result in a significant induction of *EpoR* expression, neither alone nor in combination with Sp1 [28]. Our *in vitro* data therefore indicate that regulation of *EpoR* expression by Gata4 in cardiomyocytes is a very specific mechanism that differs from *EpoR* regulation in haematopoietic and neuronal cells.

The *in vivo* models provide further evidence that Gata4 predominantly regulates *EpoR* expression in the structurally normal murine heart. Analysis of transgenic mice with inducible shRNA against *Gata4* shows significant down-regulation of *EpoR* expression, if *Gata4* expression is reduced (Fig. 4). In these transgenic mice, some Gata4 downstream targets, such as the genes encoding Bmp4 and Anf, are expressed at lower levels than in wild-type mice, whereas other Gata4 downstream targets such as the $\alpha\text{-Mhc}$ gene are normally expressed [27]. This phenomenon could be due to differences in the affinity of Gata4 to regulatory elements of the target genes. In this context it is important that Gata6, which has often been discussed to compensate for Gata4 deficiency [5, 6, 39], is not modulated in the *Gata4* shRNA expressing transgenic mice [27] and does not regulate cardiac *EpoR* expression (Figs 2, 3 and 5).

Our data confirming the activation of cardiac *EpoR* expression by Gata4 in the mouse model of doxorubicin-induced cardiomyopathy are very important for the future translation into strategies for cardioprotection. The high clinical impact is given by the fact that GATA4 mRNA and protein levels are about twofold lower in failing

human hearts than in healthy cardiac tissues [40]. Doxorubicin, which is broadly used for cancer treatment, causes irreversible degenerative cardiomyopathy and congestive heart failure [41], which is associated with Gata4 depletion in wild-type mice [3, 7, 42]. Indeed, Gata4 depletion resulted in a significant down-regulation of *EpoR* expression, while during the recovery period *EpoR* mRNA levels normalized with restored Gata4 levels. The specificity of this mechanism is supported by results on the α -Mhc expression as *bona fide* control (Fig. 5). Of note, in doxorubicin-induced cardiomyopathy Gata4 can be experimentally restored and Gata4 phosphorylation can be increased by rEpo treatment [3, 4]. This process is associated with the induction of the Erk/MAPK signalling pathway [3]. Activation of this pathway stimulates nuclear translocation of the Gata4 protein and increases Gata4 binding to regulatory DNA elements of downstream target genes [35]. Furthermore, activation of the MAPK cascade leads to the phosphorylation of Gata4, which potentiates its activity and direct, Epo-independent, cardioprotective effects [7, 42–44]. Thus, we propose that rEpo may induce *EpoR* expression in cardiomyocytes by stimulating *Gata4* expression and its transcriptional activity, at least under circumstances of Gata4 depletion. For clinical concepts performed with rEpo for cardioprotection it may be important to elucidate whether GATA4 also activates *EpoR* expression in endothelial cells [10, 45, 46].

In conclusion, we demonstrate that Gata4 is a critical component for normal *EpoR* expression in cardiomyocytes. Under clinical conditions, such as cardiomyopathy due to cancer treatment with anthracyclines or ischaemia/reperfusion injury [3, 44], down-regulation of Gata4 or Sp1 may limit the accessibility of the *EpoR* for binding of rEpo. This may explain some limitations in the first clinical trials performed with rEpo or ESA for cardioprotection in human beings [10, 46]. Thus, restoring Gata4 levels under conditions of cardiac failure may be important for future clinical concepts in using rEpo or other ESA for cardioprotection. In general, our study highlights the fundamental role of Gata4 in regulating myocardial function and recovery.

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Conflict of interest

The authors declare no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. 1(A) *EpoR* promoter constructs used for reporter gene assays highlighting the minimal promoter as well as enhancer and inhibitor elements as previously identified in haematopoietic progenitors, bone marrow stroma cells, or fibroblasts (Yousoufian H *et al.*, *Mol Cell Biol.* 1993; 13: 98–104, and Yousoufian H. *Blood.* 1994; 83: 1428–35). Numbers indicate the position of the nucleotides relative to the transcription start site and refer to various lengths of the reporter gene constructs. The –4900/+100 constructs represents the 5 kb murine *EpoR* promoter region cloned into the luciferase reporter plasmid pGL2. The truncated promoter regions –1742/+100 and *mEpoRpro* are also illustrated (see also Fig. 1 of the main manuscript). **(B)** Reporter gene activities after transfection of the 5 kb *EpoR* promoter construct, the truncated promoter constructs, or pGL2 into murine HL-1 cardiomyocytes. Firefly luciferase activities were normalized to the corresponding Renilla luciferase values and protein concentration ($n = 3$ independent experiments; ** $P < 0.01$; * $P < 0.05$).

Fig. 2 Developmental expression profile of *Epo*, *EpoR* and candidate factors for *EpoR* regulation in the heart of wild-type mice and murine HL-1 cardiomyocytes.

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