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 ischaemia-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 derivates 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...
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<sup>−/−</sup>) or the EpoR (EpoR<sup>−/−</sup>) genes [11, 12]. Both Epo<sup>−/−</sup> and EpoR<sup>−/−</sup> embryos suffer from ventricular hypoplasia, epicardial 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<sup>−/−</sup> 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 [19]. 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 TRiZo® (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
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‘-gttccctggggtgtcaggctgtcctgc-3′; −44/−14 GC-5′-cagcgcgcagtagtgccgccccccgccccctcctga-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/B6 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.
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 Δ1mEpoRpro (nt-234/+100) showed a reduction of activity to 60 ± 9.7%, and a further truncation (Δ2mEpoRpro, nt-44/+100) reduced reporter gene activity to 42 ± 2.2%. Δ3mEpoRpro (nt-8/+100), which contained only the 5′-untranslated region, showed very low activity (11 ± 1.9%) compared to mEpoRpro, and was similar to the empty pGL2 basic vector (7 ± 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 ± 6.4%). Mutation of the GATA-site showed a moderate reduction in luciferase activity (down to 72 ± 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
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.

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 ± 6% (Fig. 3B). For Sp1 knockdown, we transfected a short hairpin Sp1 plasmid or the corresponding scramble control. The Sp1
knockdown was verified by Western blot. Q-PCR analysis showed a clear reduction in EpoR mRNA to 55% (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 ± 4.5% of the level in control mice (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 αMHC, 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 ± 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 knockdowns were verified by immunoblot. 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.

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.

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).
significantly decreased (to 19 ± 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 α-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-E in the minimal EpoR promoter
(Fig. 2A). This suggests that accessibility of Gata4 may be impor-
tant 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 neu-
ronal 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 expres-
sion 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 expres-
sion, 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 α-Mhc gene
are normally expressed [27]. This phenomenon could be due to dif-
fences 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 (Youssoufian H et al., Mol Cell Biol. 1993; 13: 98–104, and Youssoufian 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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