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RESEARCH ARTICLE

EGF is required for cardiac differentiation of P19CL6 cells through interaction with GATA-4 in a time- and dose-dependent manner

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Abstract The regulation of cardiac differentiation is critical for maintaining normal cardiac development and function. The precise mechanisms whereby cardiac differentiation is regulated remain uncertain. Here, we have identified a GATA-4 target, EGF, which is essential for cardiogenesis and regulates cardiac differentiation in a dose- and time-dependent manner. Moreover, EGF demonstrates functional interaction with GATA-4 in inducing the cardiac differentiation of P19CL6 cells in a time- and dose-dependent manner. Biochemically, GATA-4 forms a complex with STAT3 to bind to the EGF promoter in response to EGF stimulation and cooperatively activate the EGF promoter. Functionally, the cooperation during EGF activation results in the subsequent activation of cyclin D1 expression, which partly accounts for the lack of additional induction of cardiac differentiation by the GATA-4/STAT3 complex. Thus, we propose a model in which the regulatory cascade of cardiac differentiation involves GATA-4, EGF, and cyclin D1.

Keywords Cardiogenesis · Sarcomeric myosin heavy chain · Pluripotent mouse embryonal carcinoma cells · Signaling transduction · Gene regulation

Introduction

The heart is derived from the mesoderm, and then the regions of anterior lateral mesoderm form a cardiac crescent as the primary heart field, which is specified during gastrulation, and subsequently develops into a linear heart

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tube. Next, the heart tube elongates and bends to the right, and eventually forms a functional four-chambered heart. In these stages of development, the specification of mesodermal progenitors to cardiomyocytes, (i.e., cardiac differentiation) is very important [29, 41]. Indeed, deregulation of cardiac differentiation often leads to heart disease. For instance, abnormal cardiomyocyte differentiation can lead to congenital heart malformations [14]. Due to the importance of cardiac differentiation in heart formation, the underlying molecular mechanisms by which cardiac differentiation are regulated have been intensively studied. For instance, cardiac differentiation pathways regulated by transcription factors, chromatin-remodeling proteins, and miRNAs, including GATA-4, Brg1 and miR-1, are some of the most studied regulatory events [14, 16, 37]. Many recent studies suggest that the cell cycle regulatory protein cyclin D1 plays a key role in cardiac differentiation [29, 30, 39].

The cyclin D1 protein is a D-type cyclin and regulates cell cycle progression by activating cyclin-dependent kinases (CDKs). Once induced, cyclin D1 associates with CDK4 and CDK6 to regulate down-stream targets, including retinoblastoma tumor-suppressor protein, Rb, and Rb-related proteins p107 and p130, allowing the cell to enter the cell cycle [39]. Most notably, the cyclin D1 protein is highly expressed during all stages of embryonic cardiac development and functions as a regulator of cardiomyocyte proliferation and differentiation in a coordinated manner [29, 39]. Previous studies found that cyclin D1 is induced by mitogens [29], and a recent study further provided evidence that the transcription factor KLF13 and the glycogen synthase kinase-3β (GSK-3β) act as regulators of cyclin D1 [19, 20, 30]. Most notably, cyclin D1 and a key cardiac transcription factor, GATA-4, are part of a negative feedback loop and regulate cardiac development [29], implying that GATA-4 also plays an important role in regulating cyclin D1-mediated cardiomyocyte differentiation.

GATA-4 is a zinc-finger transcription factor and is highly expressed in the heart during the development of the precardiogenic splanchnic mesoderm [4, 29]. GATA-4 has been implicated in the regulation of cardiac gene expression during heart development [23]. The cardiac genes regulated by GATA-4 have been well studied, with most of the attention having focused on several structural and regulatory genes. In a well-established model, GATA-4 activates the expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α- and β-myosin heavy chain (MHC), and cardiac troponin-I (cTnI), and subsequently regulates cardiac proliferation, differentiation, and survival [1, 3, 15, 35, 40]. Recent work demonstrated that GATA-4 activates the expression of NKX2.5, Bcl2, insulin-like growth factor-binding protein (Igfbp) 4, cyclin D2, and Cdk4 [3, 22, 25, 33]. Collectively, these studies have provided compelling evidence suggesting that GATA-4 is the key modulator of heart development.

In contrast to these structural and regulatory genes, relatively little is known about the mechanisms whereby GATA-4 regulates extracellular signals, such as cytokines and growth factors, during cardiomyocyte differentiation. Epidermal growth factor (EGF) is a well-characterized mitogen with roles in epithelial–mesenchymal transition, cell invasion, migration, proliferation, differentiation, morphogenesis, and apoptosis [2, 5, 7, 8, 10, 34]. In the heart, EGF not only plays an important role in cardiac differentiation and development but also provides cardioprotection against low-flow ischemia-induced injury [11, 26]. Thus, understanding the mechanism of transcriptional regulation of EGF is an important goal. However, relatively little is known about the mechanisms by which EGF is regulated. Han et al. [13] demonstrated that high glucose levels activate the expression of EGF in pancreatic cancer cells. Later studies found that hypoxia regulates EGF expression in human hearts and cultured cardiomyocytes [28]. Interestingly, EGF induces the differentiation of skeletal muscle-derived CD34(–)/45(–) stem cells into cardiomyocytes that express GATA-4 [38], suggesting that GATA-4 may be involved EGF-mediated cardiac differentiation.

Given the role of EGF in cardiac differentiation through the activation of GATA-4 expression, we asked whether GATA-4 regulates EGF. Specifically, we investigated the regulatory mechanisms involved in this regulation during cardiac differentiation. We describe a novel mechanism by which GATA-4 activates EGF expression and response to EGF signaling to regulate cardiac differentiation of P19CL6 cells. Moreover, GATA-4 protein directly interacts with signal transducers and activators of transcription 3 (STAT3) and cooperatively regulates EGF expression to modulate cardiac differentiation. Our findings implicate EGF as a key target in GATA-4-mediated gene expression, leading us to propose a molecular mechanism that mediates cardiac differentiation via the GATA-4/EGF/cyclin D1 pathway.

Materials and methods

Plasmids

Different deletions of GATA-4 were cloned into the *Kpn*I/*Eco*RI sites of the pcDNA 3.1(+) vector (Invitrogen, Carlsbad, CA, USA) with a 3× Flag sequence to produce in vitro-transcribed/translated proteins. GST-tagged wild-type and mutant STAT3 vectors were generated using PCR-mediated amplification and cloned into the *Bam*HI/

*Xho*I sites of the pGEX-4T-3 vector (Amersham Pharmacia, Piscataway, NJ, USA). The same STAT3 inserts were also cloned into the *Bam*HI/*Xho*I sites of the pcDNA 3.1(+) vector for expression in mammalian cells. The two rat EGF promoter sequences (−1,110 to +95 bp and −2,066 to +373 bp) were amplified from rat genomic DNA and cloned into the *Kpn*I/*Nco*I and *Kpn*I/*Xho*I sites of the pGL3 basic vector (Promega, Madison, WI, USA), respectively. The promoter reporter construct containing a mutation at the GATA-4 binding site was amplified from the EGF promoter sequence and cloned into the *Kpn*I/*Nco*I sites of the pGL3 basic vector (Promega, Madison, WI, USA). The GATA-4 RNAi and EGF RNAi constructs were designed according to the pSilencer neo™ Instruction Manual (Ambion, Foster, CA, USA) as described previously [46], and siRNA-negative control has no homology to any known mammalian gene. All constructs were confirmed by DNA sequencing. The sequences of the primers are listed in the supplemental table.

Antibodies

The following commercial antibodies were used: anti-GATA-4 (ab134057, Abcam), anti- Islet-1 (ab109517, Abcam), anti-MEF2C (ab64644, Abcam), anti-STAT3 (sc-8019, Santa Cruz), anti-cyclin D1 (sc-753, Santa Cruz), anti-MHC (MF-20, Developmental Studies Hybridoma Bank, IA, USA), and anti-α-tubulin (sc-32293, Santa Cruz). Protein A/G plus agarose (sc-2003) was also obtained from Santa Cruz.

Cell culture, transfection, and differentiation

P19CL6 cells were grown in Minimum Essential Medium Alpha Medium (α-MEM, GIBCO) supplemented with 10 % fetal bovine serum (FBS), and NIH3T3 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10 % FBS. Transfections were carried out 24 h after plating cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, for NIH3T3 cells, the EGF promoter reporter construct or the promoter reporter construct containing a mutation at the GATA-4 binding site was transiently transfected with GATA-4 and/or STAT3 expression vectors. At 36 h post-transfection, the cells were harvested, and luciferase activity was measured with a Berthold LB960 luminometer. The amount of reporter was maintained at 1 μg per well of a 12-well plate, and the amount of DNA was kept constant using the empty expression vector.

To construct cells stably expressing GATA-4 and/or STAT3, P19CL6 cells were transfected with the empty pcDNA3.1(+) vector (G418-resistant), pcDNA3-GATA-4, and/or pcDNA3-STAT3 using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Twenty-four hours later, the cells were treated with 400 μg/ml G418 (Amersham Pharmacia Biotech). G418-resistant colonies were selected after 2 weeks.

To induce the differentiation of P19CL6 cells into cardiomyocytes, P19CL6 cells were plated at a density of 1.8×10^5 cells/well in a six-well plate with α-MEM containing 1 % dimethyl sulfoxide (DMSO) for 18 days. The media was changed every 2 days. Days of differentiation were numbered consecutively after the first day of the DMSO treatment (i.e., day 0). For the GATA-4 and EGF siRNA-mediated knockdowns in P19CL6 cells, siRNAs were transfected on day 2 of differentiation. The siRNA sequences are listed in supplemental table.

Real-time PCR

Real-time PCR was performed as previously described [45]. RNA was extracted from the ventricles of GATA-4+/− mice and P19CL6 cells using the TRIzol method (Invitrogen). First-strand cDNAs were synthesized according to the standard protocol (Qiagen) and were then used as the template in subsequent real-time PCR reactions, as described previously [45]. A comparative quantification method was used and the mRNA levels were normalized to those of ribosomal protein S16. The sequences of the primers are listed in supplemental table.

Western-blot analysis

Western blotting was performed using cell lysates from P19CL6 cells according to standard protocols, as previously described [45]. Briefly, proteins from differently treated P19CL6 cells or the cardiac differentiated P19CL6 cells were separated by SDS-PAGE, transferred to PVDF membranes and subjected to immunoblotting using antibodies specific for the sarcomeric MHC, GATA-4, STAT3, cyclin D1, MEF2C, Islet-1, or α-tubulin and were visualized using a standard ECL protocol (Pierce, Rockford, IL, USA).

GST pull-down assays

The GST fusion proteins were produced in bacteria according to the manufacturer's instructions. Flag-tagged luciferase, GATA-4, and different GATA-4 deletions were produced in vitro using a TNT T7 Quick coupled transcription/translation system (Promega). The resultant proteins were incubated with the GST protein, the GST-STAT3 protein or the GST-STAT3 deletions and were then immobilized on glutathione-Sepharose beads in binding buffer [20 mM HEPES, (pH 7.5), 75 mM KCl, 0.1 mM

ethylenediaminetetraacetic acid, 2.5 mM MgCl2, 0.05 % Nonidet P-40, 10 % glycerol, 2 mM DTT, 1 mg/ml bovine serum albumin, and 0.1 mM phenylmethylsulfonyl fluoride] for 14–16 h at 4 °C. Proteins were then eluted after being washed three times with binding buffer without bovine serum albumin and analyzed by immunoblotting with an anti-Flag antibody (Sigma).

Co-immunoprecipitation

The GATA-4 and/or STAT3 vector were transiently transfected into 293T cells (ATCC). Twenty-four hours later, nuclear extracts were prepared as previously described [45]. Co-immunoprecipitation reactions were performed with 200 μg of nuclear extracts using 4 μg GATA-4 antibody (Santa Cruz, CA, USA) in 300 μl binding buffer. Protein A/G plus agarose (Santa Cruz) was used to capture the bound immunocomplexes, and the complexes were then analyzed by immunoblotting with antibodies against STAT3 (Santa Cruz).

Chromatin immunoprecipitation and real-time PCR (ChIP q-PCR) analysis

ChIP assays were performed as previously described [45]. Briefly, 16 million P19CL6 cells or cells treated with 10 ng/ml EGF for 2 days were cross-linked with 1 % formaldehyde at 4 °C for 15 min on day 16 of cardiac differentiation. Glycine (125 mM) was used to stop the cross-linking reaction. Cells were harvested and sonicated to achieve fragments of approximately 600 bp. Next, the chromatin lysate was immunoprecipitated using antibodies against GATA-4, STAT3, MEF2C, or goat IgG (Santa Cruz). Immunoprecipitated chromatin was reverse cross-linked and purified with the QIAquick PCR purification kit (Qiagen) and analyzed by real-time PCR using a Quantitect SYBR green PCR kit (Qiagen) in an ABI 7500 fast QPCR System (ABI). The sequences of the primers are listed in the supplemental table.

Histology

E15.5 embryos were fixed overnight at 4 °C in 4 % paraformaldehyde, dehydrated through graded ethanol series, embedded in paraffin, and sectioned at 4-μm intervals. Heart sections were stained with Masson's trichrome using standard procedures to visualize defects.

Statistics

The data are reported as the mean ± SEM. Student's unpaired *t* tests were used to compare two groups. In all cases, differences were considered to be statistically significant when $p < 0.05$.

Results

EGF is a target of GATA-4

GATA-4 is critical in cardiogenesis [3, 33]. To further determine the roles of GATA-4 in heart development, we performed histological analyses on E15.5 Gata4+/− heterozygotes. As shown in Fig. 1a, ventricular septal defect (VSD) as well as myocardial thinning was evident in at least 43 % of embryos, which revealed profound structural cardiac defects. To determine the potential mechanism for these defects, we examined total mRNA levels in the ventricles of GATA-4+/− mice to identify potential targets of GATA-4. Unexpectedly, we found that the mRNA level of EGF was down-regulated in the ventricles of E12.5 (Fig. 1b, upper), E14.5 (Fig. 1b, middle), 2-day-old (Fig. 1b, lower), 50-day-old (Fig. 1c, left), and 120-day-old (Fig. 1c, right) GATA-4+/− mice, compared with the level in wild-type mice (Fig. 1b–d). This finding suggested that EGF might be a target of GATA-4. In addition, as shown in Fig. 1e, the co-transfection of a 2,439- or 1,205-bp rat EGF promoter construct with a GATA-4 expression vector in NIH3T3 cells resulted in the activation of EGF promoter in a dose-dependent manner, while mutation of the GATA-4-binding sites in the EGF promoter abrogated the activation, indicating that EGF is a down-stream target of GATA-4. Furthermore, to test whether GATA-4 binds to the EGF promoter in vivo, chromatin immunoprecipitation (ChIP) was performed on day 16 of cardiac differentiation of P19CL6 cells using a GATA-4 antibody. The results indicated that the EGF promoter region (−1,900 to −1751 bp and −34 to −160 bp) was specifically occupied by a GATA-4 antibody and not by the IgG control antibody (Fig. 1f), whereas negative control locus that does not bind to the transcription factor cannot be occupied by a GATA-4 antibody. Thus, we confirmed that EGF is a bona fide target gene of GATA-4.

EGF is essential for cardiogenesis

Heart development is a complex process including the specification of cardiomyocytes, the determination of the cardiac field, the differentiation of cardiac precursor cells, and the formation of the specific anatomical structure. Structural cardiac defects maybe result from the loss of myocardial specification and differentiation of cardiac precursor cells [6, 9]. P19CL6 cells, a clonal derivative line from P19 embryonic teratocarcinoma cells, have been used as an in vitro model system to study early heart differentiation because these cells can more efficiently differentiate into beating cardiomyocytes than parental P19 cells in the presence of 1 % dimethyl sulfoxide (DMSO) [6]. To examine whether GATA-4 plays roles in cardiomyocyte

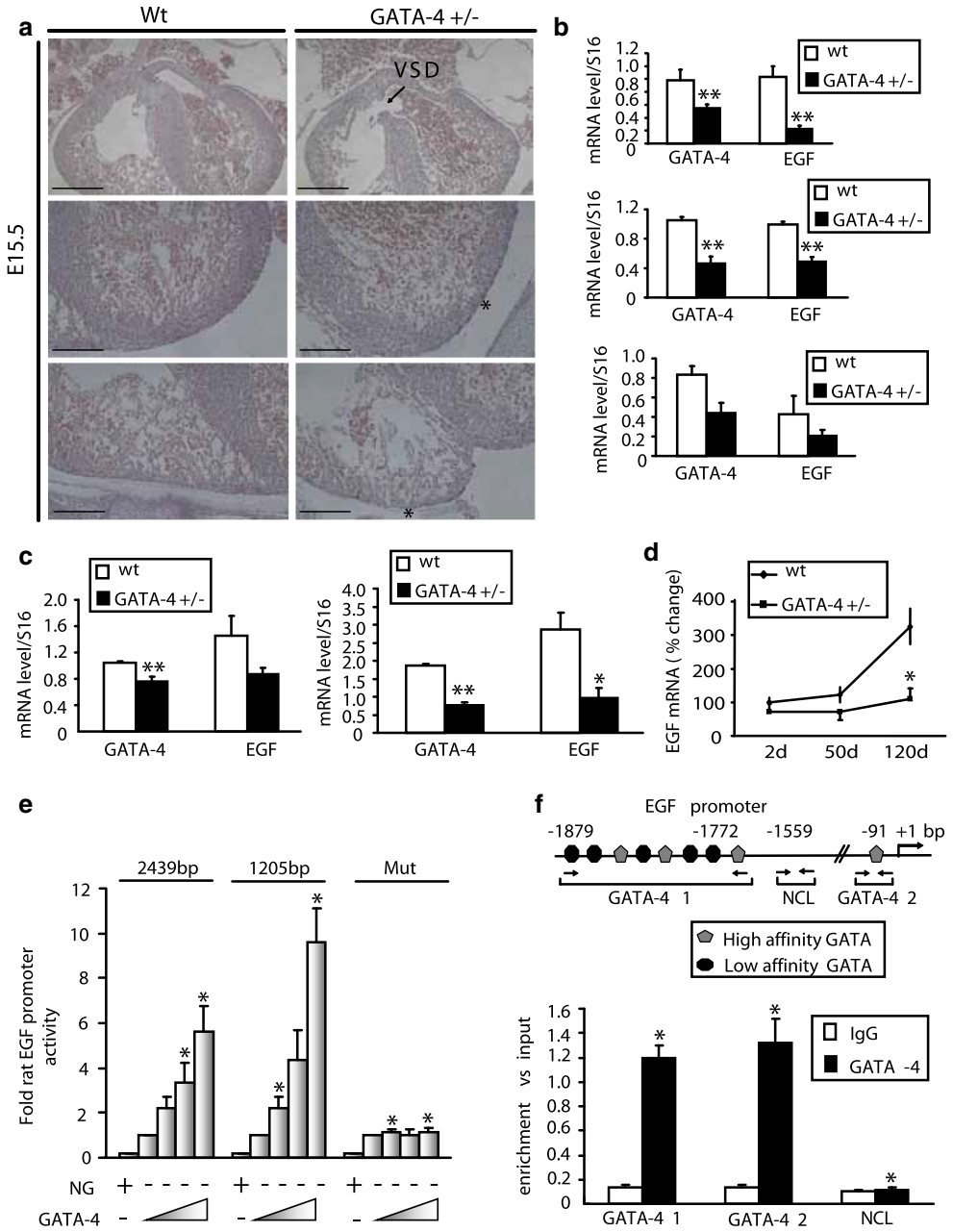


Fig. 1 Regulation of EGF expression by GATA-4. **a** Cardiac defects in Gata4+/− heterozygotes. Trichrome staining of transverse sections of E15.5 embryos. Transverse section through the heart showing ventricular septal defect (VSD) (*arrow* in the *upper right*) and myocardial thinning (*star* in the *right middle* and *right lower*) in Gata4+/− embryos. *Bars* 300 μm (*upper*), and *bars* 100 μm (*middle* and *lower*). **b–c** The mRNA level of epidermal growth factor (EGF) is severely down-regulated in E12.5 (**b**, *upper*), E14.5 (**b**, *middle*), 2-day-old (**b**, *lower*), 50-day-old (**c**, *left*), and 120-day-old (**c**, *right*) GATA-4+/− mice. RNA was extracted from the ventricles of GATA-4+/− mice and age-matched wild-type (wt) mice and the transcript levels for GATA-4 and EGF were determined. **d** Time courses of the changes in EGF mRNA levels in the ventricles of GATA-4+/− and wt mice. Data are mean ± SEM with 4 (GATA-4+/−) and 6 (wt) mice per group. $*P < 0.05$ vs. Wt, and $**P < 0.01$ vs. Wt. **e** GATA-4 dose-dependent activation of the EGF promoter. A rat EGF promoter luciferase construct (−2,439 and −1,205 bp) and the construct harboring a mutation in the GATA-4-binding sites (Mut) were transfected with pGL 3-basic vector (negative control, NG), 5, 10, and 25 ng of the GATA-4 expression vector in NIH3T3 cells, and luciferase activity was analyzed after 36 h. The data shown are the mean ± SEM of three independent experiments carried out in duplicate (out of 3). $*p < 0.05$ vs. control. **f** Increased occupancy of the EGF promoter by GATA-4 protein. Cardiac differentiated P19CL6 cells on day 16 were processed for ChIP analysis using the GATA-4 antibody. The immunoprecipitated DNA was analyzed by real-time PCR. Schematic representation of the EGF promoter showing the GATA elements together with the primers used in the ChIP analysis. The results shown are the mean ± SEM of three independent experiments. $*p < 0.05$ vs. IgG. *NCL* negative control locus (locus that does not bind to the transcription factor)

differentiation, we constructed a P19CL6 cell line stably expressing GATA-4 and analyzed its ability to differentiate into cardiomyocytes in the presence of 1 % DMSO. Cardiomyocyte differentiation was assessed by determining the expression of sarcomeric MHC proteins, which were detected by immunoblot using the anti-sarcomeric MHC antibody MF20 [12]. MHC was not expressed in control or GATA-4-expressing P19CL6 cells on day 0 of cardiac differentiation (Fig. 2a). However, in response to DMSO, GATA-4 expression cells induced MHC expression on day 14, compared with day 16 in the control cells, suggesting that GATA-4 promotes cell differentiation (Fig. 2a). On the other hand, knocking down GATA-4 blocked cardiomyocyte differentiation of P19CL6 cells, whereas a negative control Si RNA can maintain cardiomyocyte differentiation of P19CL6 cells (Fig. 2a). Consistent with these results, real-time PCR analysis revealed that during differentiation, compared to P19CL6 cells that were differentiated on day 0, sarcomeric α-MHC mRNA levels were significantly increased in cells expressing GATA-4, especially after day 10 of differentiation, whereas knocking down GATA-4 resulted in down-regulation of expression of α-MHC. In fact, the 7 % increase in sarcomeric α-MHC mRNA levels on day 16 in the knockdown cells was not sufficient to initiate differentiation (Fig. 2b–d). Similar results were observed for cardiac transcription factor GATA-4 mRNA levels (Fig. 2b–d). More importantly, the expression levels of EGF were up-regulated in response to GATA-4 over-expression and down-regulated in response to knock down of GATA-4 expression (Fig. 2c, d), suggesting that EGF plays a role in GATA-4-mediated cardiac differentiation.

To further determine the possible role of EGF in cardiac myogenesis, we monitored differentiation induced by DMSO with the addition of EGF at concentrations of 10, 50, or 100 ng/ml for 2, 4, or 12 days. As shown in Fig. 3, compared to the control cells (Fig. 3a, upper left), EGF treatment dramatically promoted cardiac differentiation at a concentration of 10 ng/ml for 2 and 4 days, 50 ng/ml for 2 and 4 days, and 100 ng/ml for 2 days (Fig. 3a–c). However, 4 days of treatment with EGF at 100 ng/ml only slightly induced cardiac differentiation, and 12 days of EGF treatment with the same concentration thoroughly blocked DMSO-induced cardiomyogenic differentiation (Fig. 3c). Given that the increased MHC expression could be due to the increased cardiomyocyte differentiation from P19CL6 cells, or the increased proliferation of differentiated cardiomyocytes in the culture system, so EGF induces cardiac differentiation as well as proliferation of differentiated cardiomyocytes in a dose- and time-dependent manner. Of note, the RNAi-mediated knockdown of EGF expression also prevented cardiac differentiation (Fig. 3d), suggesting that EGF is required for cardiac differentiation.

To further investigate cardiac gene expression following EGF exposure during cardiomyogenic differentiation, real-time PCR analysis was performed to detect the expression of α-MHC, GATA-4, and EGF. We found that treatment with EGF profoundly increased α-MHC expression at a dosage of 10 ng/ml for 2 days or 50 ng/ml for 4 days, as shown in Fig. 3e, Figure S1A, and S1B. The α-MHC mRNA level was first increased at 4 days or 6 days after cardiac induction and the maximal level was reached after 18 days. Conversely, prolonged treatment with a high dose of EGF (100 ng/ml for 12 days) drastically decreased α-MHC levels (Figure S1C). Moreover, compared with the Si RNA-negative control, inhibition of α-MHC expression was also observed during differentiation when EGF expression was knocked down in P19CL6 cells (Figure S1D and S1E). Likewise, GATA-4 expression was profoundly increased upon administration of 10 ng/ml EGF for 2 days or 50 ng/ml for 4 days compared with levels in the untreated control cells (Fig. 3e, S1A, and S1B). In contrast, exposure to 100 ng/ml EGF for 12 days and the knockdown of EGF in P19CL6 cells failed to significantly enhance GATA-4 expression (Figure S1C and S1E), indicating that GATA-4 expression responds to EGF during P19CL6 cell differentiation in a dose- and time-dependent manner. On the other hand, expression of EGF was significantly increased upon exposure to EGF but decreased upon removal of EGF (Figure S1A–S1E). Taken together, these results suggest that EGF is essential for cardiogenesis and promotes dose- and time-dependent cardiac differentiation.

Functional interaction between EGF and GATA-4 during cardiac differentiation of P19CL6 cells in a time- and dose-dependent manner

As stated above, EGF is essential for cardiogenesis and plays a role in GATA-4-mediated cardiac differentiation. To further elucidate the functional connection between GATA-4 and EGF, we investigated what role, if any, EGF might play in cardiac differentiation induced by GATA-4. To this end, the expression of sarcomeric MHC proteins was assessed by Western blot during differentiation in GATA-4-expressing and EGF-knock down P19CL6 cells. As shown in Fig. 4a, although GATA-4 induces MHC expression (Fig. 2a, c), knocking down EGF led to the loss of MHC expression induced by GATA-4 (Fig. 4a-1), whereas knocking down RNA-negative control still maintained the MHC expression induced by GATA-4 (Fig. 4a-2). Consistent with this observation, we found that knocking down EGF in GATA-4-expressing P19CL6 cells resulted in the down-regulation of cardiac genes (α-MHC, GATA-4, and EGF), except on day 14 for α-MHC and days 4 and 6 for GATA-4 (Fig. 4b), whereas knocking down

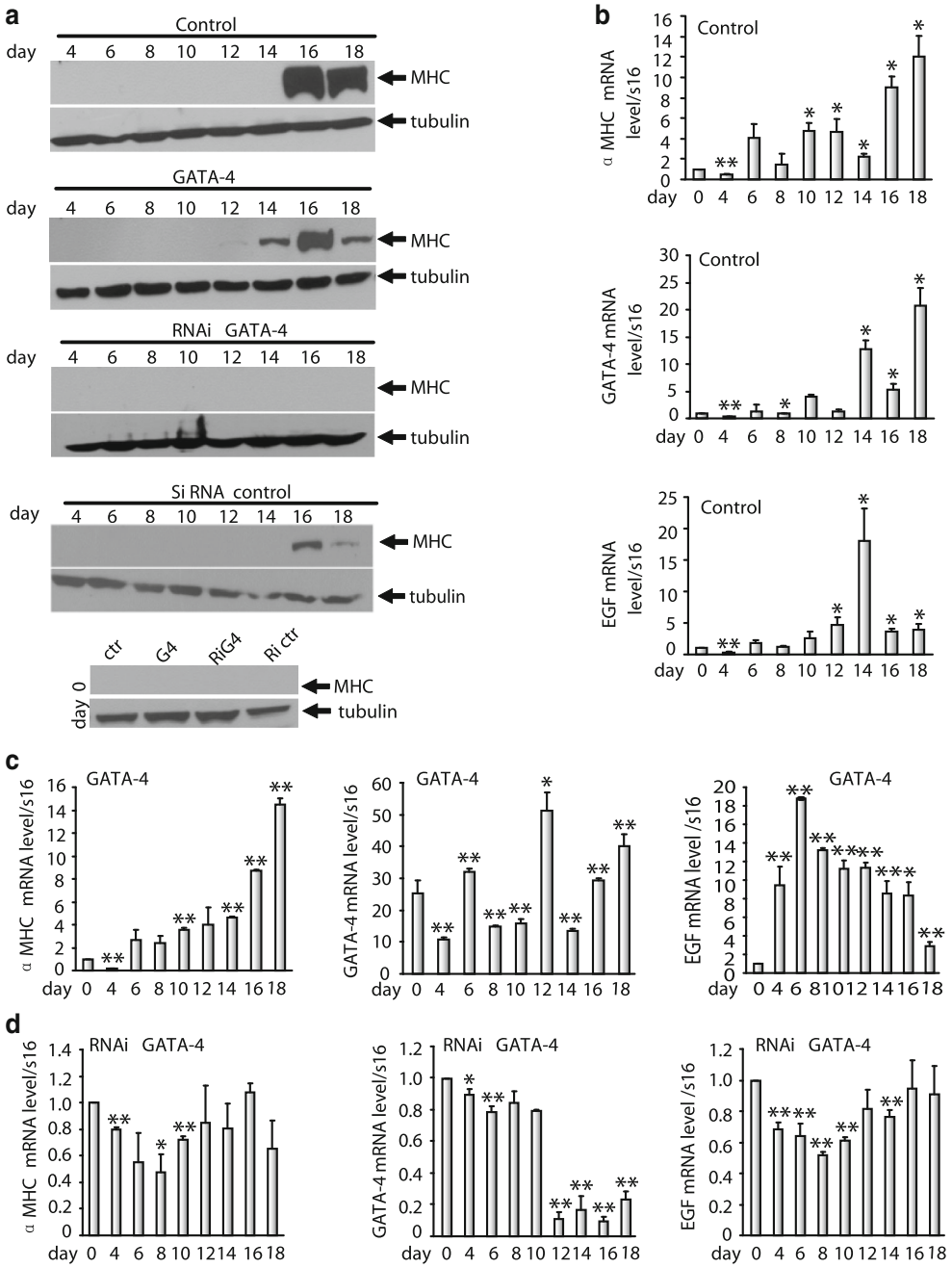


Fig. 2 GATA-4 induces the differentiation of P19CL6 cells into cardiomyocytes. **a** The effect of GATA-4 on cardiomyocyte differentiation was assessed using MF20 immunoblotting in P19CL6 cell lysates from cells stably transfected with pCDNA3.1 (+) empty vector (*upper*), the GATA-4 expression vector, the GATA-4 knockdown vector, and Si RNA negative control vector (*lower*). The cells were cultured for 18 days in 1 % DMSO. At the indicated time points, the cell lysates were subjected to immunoblot analysis with the antibodies indicated in the figure. The cell lysates were also subjected to immunoblot analysis on day 0 (lowest, *ctr* control, *G4* GATA-4, *RiG4* RNAi-GATA-4, *Ri ctr* Si RNA control). **b** Cardiac gene expression was assessed by real-time PCR in P19CL6 cells treated with 1 % DMSO. On the indicated days, P19CL6 cells transfected with pcDNA3.1(+) empty vector were evaluated for α-MHC, GATA-4, and EGF mRNA levels. The results are the mean ± SEM. $*p < 0.05$ vs. control, and $**p < 0.01$ vs. control. **c** The mRNA levels of α-MHC, GATA-4, and EGF were analyzed on day 0 and days 4 to 18 in P19CL6 cells stably expressing GATA-4 during differentiation. The results are the mean ± SEM. $*p < 0.05$ vs. control, and $**p < 0.01$ vs. control. **d** The mRNA levels of α-MHC, GATA-4, and EGF were analyzed in P19CL6 cells with GATA-4 knock down at the indicated time points. The numbers in *y* axis stand for the activated number of target gene's mRNA level corrected by S16. The results are the mean ± SEM. $*p < 0.05$ vs. control, and $**p < 0.01$ vs. control

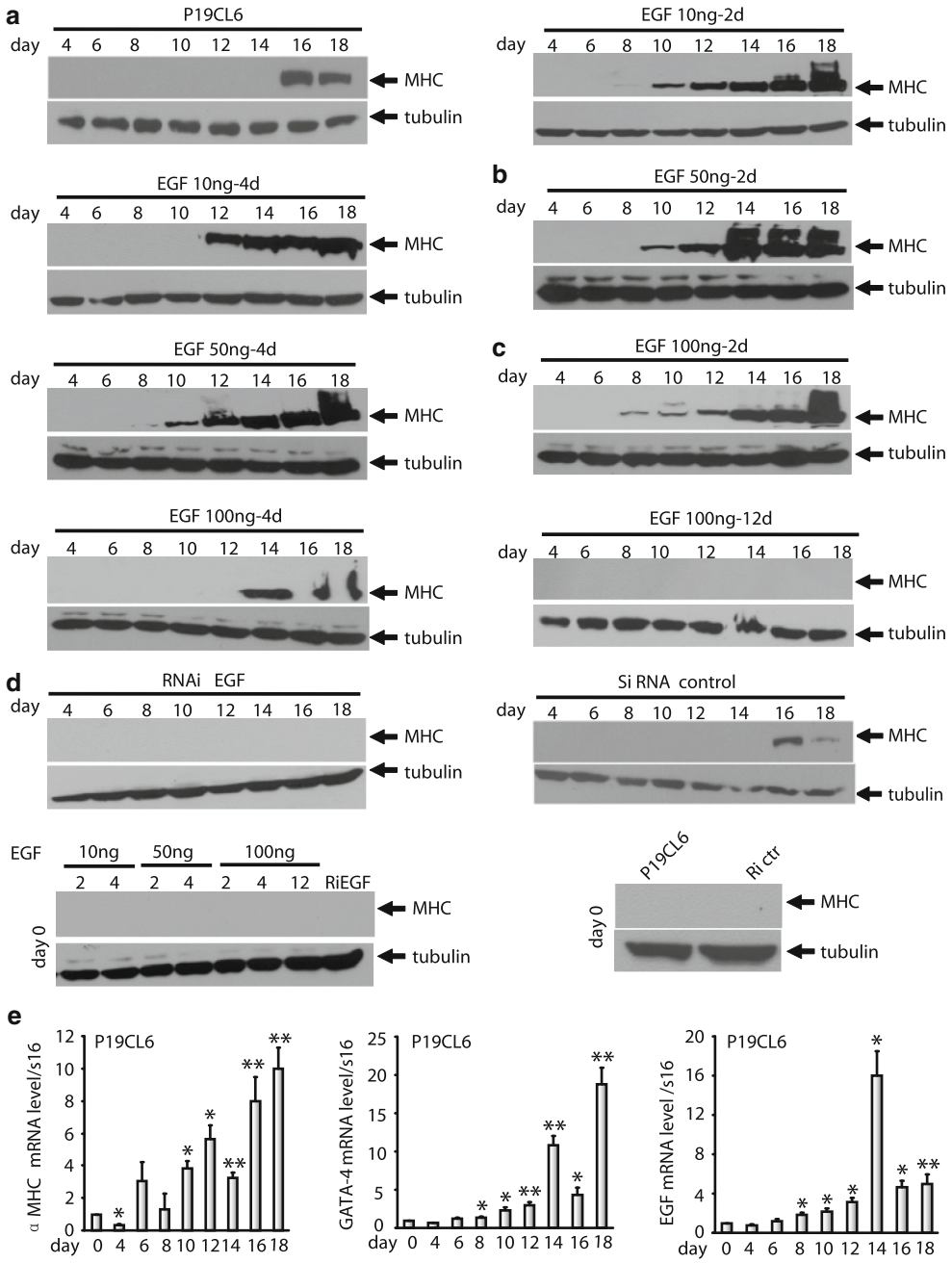


Fig. 3 EGF induces the dose- and time-dependent differentiation of P19CL6 cells into cardiomyocytes. **a** P19CL6 cells, with the specific induction of sarcomeric MHC proteins (detected by MF20 immunoblot) after exposure to 10 ng/ml EGF for 2 days (*upper right*) or 4 days (*lower left*), compared to the DMSO vehicle control (*upper left*) which did not result in detectable expression of sarcomeric MHC until day 16. **b** EGF-mediated induction of sarcomeric MHC proteins by MF20 immunoblot in P19CL6 cells upon the administration of 50 ng/ml EGF for 2 days (*upper right*) or 4 days (*lower left*). **c** Time-responsive induction of sarcomeric MHC proteins in P19CL6 cells by the administration of 100 ng/ml EGF for 2 days (*upper right*), 4 days (*lower left*), or 12 days (*lower right*). **d** Knocking down EGF (RNAi EGF) suppresses the induction of sarcomeric MHC proteins. The cell lysates were also subjected to immunoblot analysis on day 0 (*lower*). **e** Cardiac gene expression was assessed by real-time PCR in P19CL6 cells treated with 1 % DMSO. The results are the mean ± SEM. $*p < 0.05$ vs. control, and $**p < 0.01$ vs. control

RNA-negative control in GATA-4-expressing P19CL6 cells can maintain the up-regulation of cardiac genes (α-MHC, GATA-4, and EGF) (Figure S2A). On days 4 and 6, GATA-4 expression was increased 2.4- and 3.5-fold, respectively, compared to control cells, whereas EGF expression remained down-regulated during differentiation from day 4 to day 18. This lower expression level of EGF cannot maintain sufficient activation of α-MHC, so the

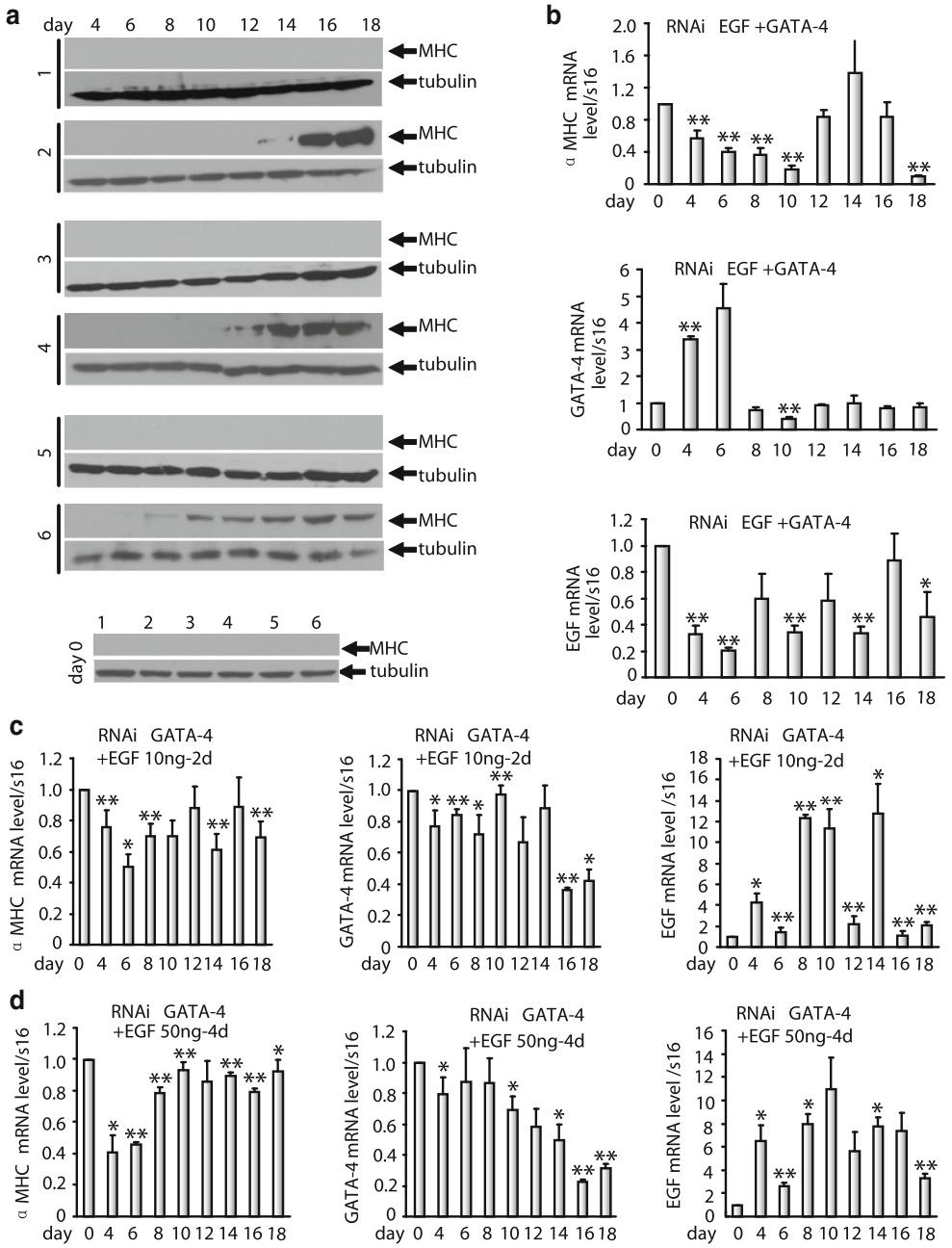


Fig. 4 The inhibition of cardiac differentiation by EGF RNAi or GATA-4 RNAi cannot be rescued by the overexpression of GATA-4 or by EGF treatment. **a** The expression of sarcomeric MHC was inhibited in P19CL6 cells over-expressing GATA-4 and the EGF RNAi cells (**1**), and in the GATA-4 RNAi cells exposed to 10 ng/ml EGF for 2 days (**3**) or 50 ng/ml EGF for 4 days (**5**). The cell lysates from the cells described above on day 0 were also subjected to immunoblot analysis (*1* RNAi EGF + GATA-4, *2* Si RNA control + GATA-4, *3* RNAi GATA-4 + EGF 10 ng-2d, *4* Si RNA control + EGF 10 ng-2d, *5* RNAi GATA-4 + EGF 50 ng-4d, *6* Si RNA control + EGF 50 ng-4d). **b–d** α-MHC, GATA-4 and EGF mRNA levels were determined by real-time PCR analysis in P19CL6 cells treated as described in **a**. The results are the mean ± SEM of three independent experiments. *$p < 0.05$ vs. control, and **$p < 0.01$ vs. control

depletion of EGF in P19CL6 cells stably expressing GATA-4 inhibited cardiac differentiation (Fig. 4a, b). Analogously, GATA-4 knockdown impaired EGF-stimulated cardiac differentiation [Fig. 4a (3–6), c–e; Figure S2 B–C]. Although EGF expression was activated by treatment with 10 ng/ml EGF for 2 days or 50 ng/ml for 4 days, the knockdown of cardiac transcription factor GATA-4 resulted in the deactivation of the cardiac marker gene MHC, indicating a block of cardiac differentiation. Together, these data indicate that GATA-4 and EGF are

functionally interdependent in promoting cardiac differentiation.

Conversely, in P19CL6 cells that over-express GATA-4, the administration of either 10 ng/ml EGF for 2 days or 50 ng/ml EGF for 4 days did not further promote cardiac differentiation (Fig. 5) compared to GATA-4 overexpression or EGF treatment alone. Although both GATA-4 and EGF were activated early during differentiation, sufficient activation of MHC was observed through day 16, suggesting that the functional interaction between GATA-4 and EGF in cardiac differentiation is time- and dose-dependent and might be involved in more complicated mechanisms, such as EGF signal transduction.

STAT3 directly interacts with GATA-4

STAT3 is a downstream mediator of EGF signaling and is crucial for the cardiac differentiation of P19CL6 cells

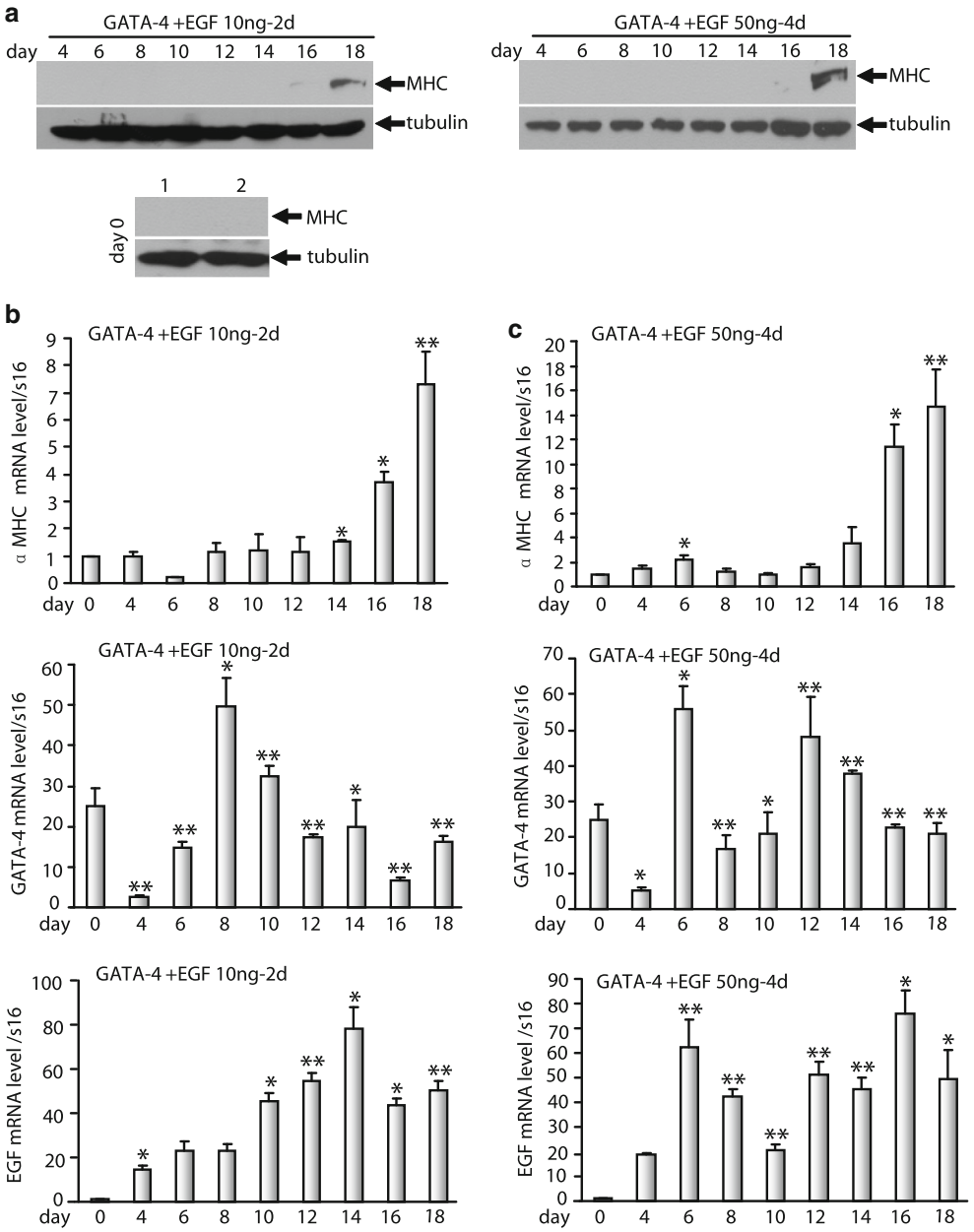


Fig. 5 GATA-4 does not enhance EGF-induced cardiac myogenesis. **a** Stably expressed GATA-4 suppresses the induction of sarcomeric MHC protein in P19CL6 cells by the administration of 10 ng/ml EGF for 2 days (*upper left*) or 50 ng/ml EGF for 4 days (*upper right*), compared to cells treated with only EGF (Fig. 3), which expressed sarcomeric MHC protein on day 10. The cell lysates of the cells described above on day 0 were also subjected to immunoblot analysis (*left lower*, *lane 1* GATA-4 + EGF 10 ng-2d; *lane 2* GATA-4 + EGF 50 ng-4d). **b**, **c** The induction of cardiac-specific markers was analyzed by real-time PCR. The results are the mean ± SEM of three independent experiments. $*p < 0.05$ vs. control, and $**p < 0.01$ vs. control

[36]. To verify whether STAT3 associates with GATA-4 in response to EGF stimulation, chromatin immunoprecipitation real-time PCR (ChIP-qPCR) experiments were performed using primers spanning the GATA-4 binding region (from −1,900 to −1,751 bp or from −160 to −34 bp) as well as transcriptional factor no binding region (−1,560 to −1,698 bp) of the EGF promoter on day 16 of cardiac differentiation of P19CL6 cells treated or untreated with 10 ng/ml EGF for 2 days. The results indicated that STAT3 was present on the region of GATA-4 binding sites not negative control locus under both basal and EGF-stimulated conditions (Fig. 6a, upper). We further confirmed that STAT3 is also present on the region of STAT3 binding site (from −1,260 to −1,109 bp) of the EGF promoter and that upon EGF stimulation there is an increase in STAT3 occupancy (Fig. 6a, lower). Interestingly, GATA-4 has been shown to occupy the region of STAT3 site following EGF treatment (Fig. 6a, lower), whereas neither STAT3 nor GATA-4 occupy the negative control locus under both basal and EGF-stimulated conditions (Fig. 6a, lower). Collectively, these data support a model in which GATA-4 recruits STAT3 to the EGF promoter independent of EGF stimulation. In agreement with these observations, co-immunoprecipitation assays showed a specific association between STAT3 and precipitated GATA-4 proteins (Fig. 6b). Thus, we confirmed GATA-4 as a bona fide STAT3-interacting protein.

To further identify the structural determinants required for the STAT3-GATA-4 interaction, we generated several deletion mutants of GATA-4 and STAT3 and performed GST pull-down assays with purified GST-STAT3 or various deletion mutant fusion proteins and in vitro transcribed and translated Flag-GATA-4 proteins (Fig. 6c, d). GATA-4 was found to interact strongly with GST-STAT3, indicating that they interact directly (Fig. 6c). This interaction requires the Src homology 2 (SH2) domain of STAT3 because $STAT3_{1–680}$ showed a strong interaction with GATA-4, but $STAT3_{1–582}$, which lacks the SH2 domain, does not interact with GATA-4 (Fig. 6c). To dissect the GATA-4 domain(s) responsible for its interaction with STAT3, full-length Flag-GATA-4 and various deletions were produced using an in vitro transcription and translation system. When incubated with GST-STAT3, only the full-length GATA-4 (1–443 aa), $GATA\text{-}4_{1–312}$, and $GATA\text{-}4_{1–251}$ were able to interact with STAT3. Interestingly, $GATA\text{-}4_{208–443}$, which lacks the N-terminal transactivation domain (TAD), showed a weak interaction with STAT3. When the first zinc finger of $GATA\text{-}4_{208–443}$ was deleted, generating $GATA\text{-}4_{245–443}$, the interaction was fully abolished (Fig. 6d), indicating that the N-terminal TAD and the first zinc finger of GATA-4 are crucial for mediating its interaction with STAT3.

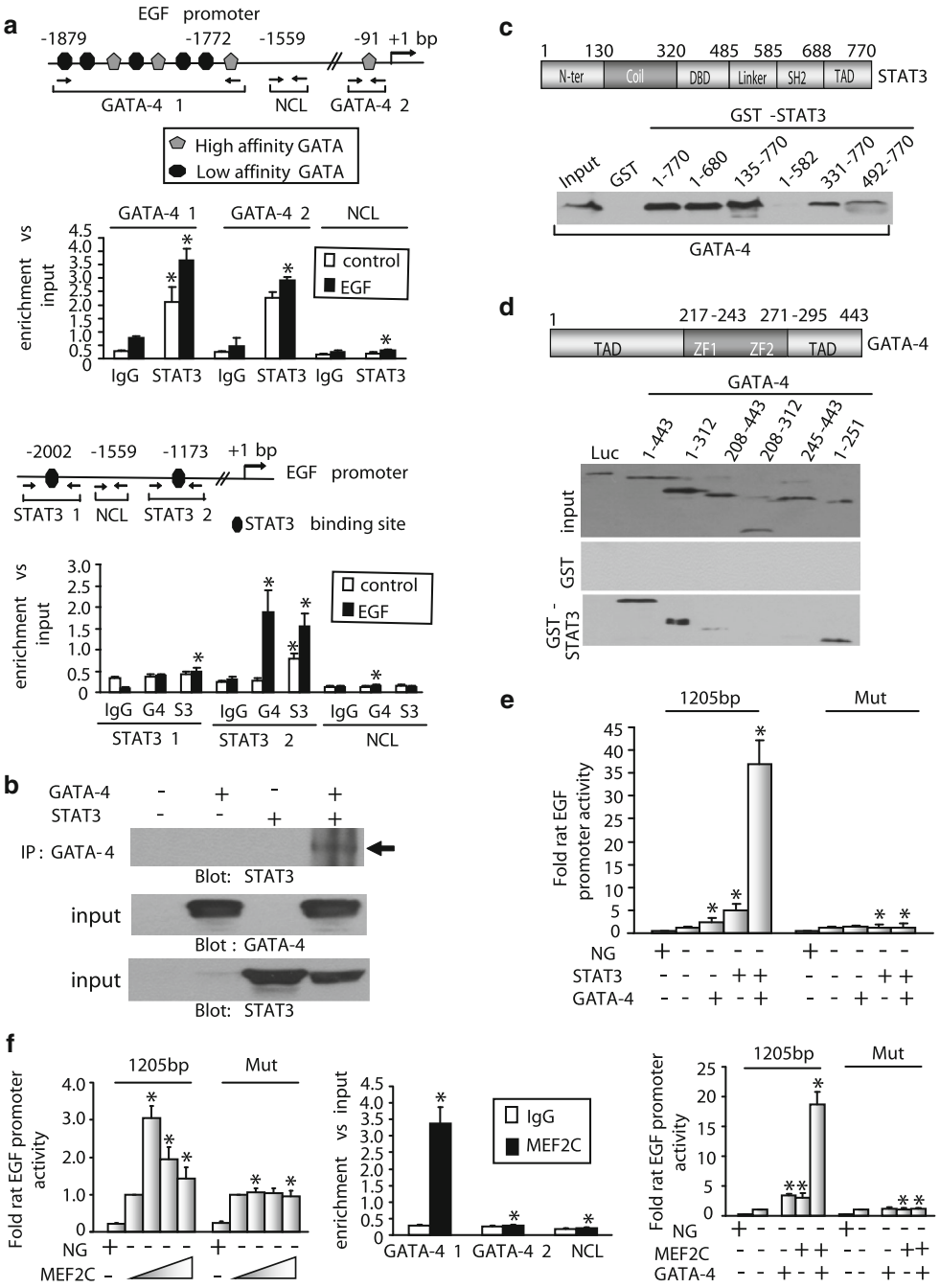
To determine whether the interaction between GATA-4 and STAT3 results in functional cooperation, transient transfection experiments were performed in NIH3T3 cells using a reporter plasmid containing the rat EGF promoter and a GATA-4 and/or STAT3 expression vector. Indeed, GATA-4 and STAT3 cooperate to activate the EGF promoter, whereas mutation of the GATA-4 binding sites in the EGF promoter abrogated the activation (Fig. 6e). Together, these data indicate that functional cooperation between GATA-4 and STAT3.

Given that myocyte enhancer factors 2C (MEF2C) is an important cardiogenic regulatory factors, and can form a protein complex with GATA-4 to synergistically activate cardiac gene transcription [27, 42], we were interested to know if GATA-4 and MEF2C can synergistically activate the EGF promoter. We found that although there is no MEF2C binding site in the EGF promoter, MEF2C still activates the EGF promoter in a dose-dependent manner (Fig. 6f, left). Furthermore, ChIP experiments demonstrate that MEF2C occupies to the promoter region of EGF in the GATA-4 binding site (Fig. 6f, middle). More importantly, there is a synergistic activation of EGF promoter between GATA-4 and MEF2C (Fig. 6f, right), suggesting that MEF2C maybe involved in the GATA4/EGF regulatory interaction.

The roles of cooperation between GATA-4 and STAT3 in cardiac differentiation

The finding that STAT3 interacts with GATA-4 prompted us to investigate the potential role of STAT3 in cardiac differentiation. To this end, we increased the STAT3 level in P19CL6 cells by stable transfection. Compared with control P19CL6 cells (Fig. 2a), cardiac differentiation in P19CL6 cells was significantly enhanced by STAT3 overexpression and was further induced upon the administration of 10 ng/ml EGF for 2 days (Fig. 7a).

To determine whether STAT3 induces cardiac differentiation in a complex with GATA-4, we tested the effect of the co-expression of STAT3 and GATA-4 on the differentiation of P19CL6 cells. As revealed in Fig. 7b, c, no co-activation of MHC expression was achieved in comparison to GATA-4 or STAT3 alone (Figs. 2a, 7a). Interestingly, EGF treatment reduced the overall level of MHC protein while having no inhibitory effect on the expression of GATA-4 or EGF (Fig. 7b, d), similar to the observation of EGF treatment in the P19CL6 cells stably expressing GATA-4 (Fig. 5). Together, these experiments indicate that the interaction between GATA-4 and STAT3 only maintains cardiac differentiation but does not stimulate it in a cooperative fashion. In addition, this maintenance of differentiation ceases in response to EGF treatment.



Cyclin D1 may account, in part, for the lack of cooperation between GATA-4 and STAT3 during cardiac differentiation of P19CL6 cells

The finding that EGF treatment suppresses MHC expression but activates GATA-4 and EGF expression during cardiac differentiation of P19CL6 cells over-expressing either GATA-4/STAT3 or GATA-4 alone raised the question of why both GATA-4 and EGF are activated but cardiac differentiation is inhibited. Previous studies have indicated that enhanced expression of cyclin D1 inhibits the differentiation of cardiomyocytes [29], and our previous studies showed that GATA-4 activates cyclin D1 transcription and expression [44]. We also found that the mRNA level of cyclin D1 was down-regulated in the ventricles of E12.5 and E14.5 GATA-4+/− mice, compared with the level in wild-type mice (Fig. 8a). Our results further demonstrate that cyclin D1 is a downstream target of GATA-4. To assess

◀**Fig. 6** GATA-4 and STAT3 interact with each other and cooperatively activate transcription of the EGF gene. **a** The increased occupancy of the EGF promoter by GATA-4/STAT3 upon EGF stimulation. The promoter occupancy of the EGF gene was evaluated by ChIP analysis. Cardiac differentiation was induced in P19CL6 cells treated or untreated with 10 ng/ml EGF for 2 days, ChIP assays were performed with an antibody against GATA-4 (G4), STAT3 (S3), or control IgG on day 16. Immunoprecipitated DNA, which revealed the enrichment of GATA-4 and STAT3 proteins on the EGF promoter, was analyzed by real-time PCR. Schematic representation of the EGF promoter showing the GATA-4 or STAT3 binding elements together with the primers used in the ChIP analysis. The results shown are the mean ± SEM of three independent experiments. $*p < 0.05$ vs. IgG. *NCL* negative control locus (locus that does not bind to the transcription factor). **b** Interactions between STAT3 and GATA-4 in vivo. Immunoblots of nuclear extracts or immunoprecipitated proteins from 293T cells transiently transfected with the GATA-4 and/or STAT3 expression vectors. **c** Interactions of STAT3 and STAT3 fragments with full-length GATA-4. GST pull-down assays were performed by incubating the in vitro translated Flag-GATA-4 with GST or GST fusion proteins containing different regions of STAT3 followed by SDS-PAGE and immunoblotting with an anti-Flag antibody. $STAT3_{1–582}$, which contains the DNA binding domain (DBD) but not the C-terminal transcription activation domain (TAD), cannot interact with GATA-4. The *top panel* shows the structure of STAT3, including an N-terminal domain, the coiled-coil domain, DNA binding domain (DBD), the linker domain, the Src homology 2 (SH2) domain, and the transcription activation domain (TAD). **d** Interactions of GATA-4 and GATA-4 fragments with full-length STAT3. Bead-immobilized GST or GST-STAT3 (1–770) fusion proteins were incubated with the indicated in vitro-translated Flag-GATA-4 (full-length or deletions), or the luciferase control, and bound proteins were analyzed by SDS-PAGE and immunoblotting with the anti-Flag antibody. The *upper panel* shows the structure of GATA-4, its transcription activation domain (TAD), and two zinc-finger domains (ZF1 and ZF2). **e** Synergistic transcriptional activation of EGF by GATA-4 and STAT3. Transient transfections were performed in NIH3T3 cells using 1,205-bp EGF promoter-driven luciferase reporter constructs or the construct harboring a mutation in the GATA-4-binding sites (Mut) with pGL 3-basic vector (negative control, NG), empty vector, or various combinations of GATA-4 and STAT3 expression vector. The data shown here are the mean ± SEM of three independent experiments. $*p < 0.05$ vs. control. **f** Synergistic transcriptional activation of EGF by GATA-4 and cardiogenic regulatory factors MEF2C. MEF2C dose-dependent activation of the EGF promoter (*left*) and increased occupancy of the EGF promoter on GATA-4 binding site (*middle*). When co-transfection with GATA-4, a synergistic transcriptional activation of EGF was observed (*right*). Briefly, a rat EGF promoter luciferase construct (−1,205 bp) and the construct harboring a mutation in the GATA-4-binding sites (Mut) were transfected with pGL 3-basic vector (negative control, NG), 100, 250, and 500 ng of the MEF2C expression vector in NIH3T3 cells, and luciferase activity was analyzed after 36 h. The data shown are the mean ± SEM of three independent experiments carried out in duplicate (out of 3). $*p < 0.05$ vs. control (*left*). *Middle* Increased occupancy of the EGF promoter by MEF2C protein. Cardiac differentiated P19CL6 cells on day 16 were processed for ChIP analysis using the MEF2C antibody. The immunoprecipitated DNA was analyzed by real-time PCR. The results shown are the mean ± SEM of three independent experiments. $*p < 0.05$ vs. IgG. *NCL* negative control locus (locus that does not bind to the transcription factor). *Right* Synergistic transcriptional activation of EGF by GATA-4 and MEF2C. Transient transfections were performed in NIH3T3 cells using 1,205-bp EGF promoter-driven luciferase reporter constructs or the construct harboring a mutation in the GATA-4-binding sites (Mut) with pGL 3-basic vector (negative control, NG), empty vector, or various combinations of GATA-4 and MEF2C expression vector. The data shown here are the mean ± SEM of three independent experiments. $*p < 0.05$ vs. control

whether cyclin D1 proteins account for the GATA-4/STAT3 upon EGF stimulation, we examined the expression level of cyclin D1 protein in control P19CL6 cells, P19CL6 cells transfected with STAT3, and/or GATA-4 expression vectors, and P19CL6 cells treated with different doses and different treatment periods of EGF. The results shown in Fig. 8 indicate that GATA-4 alone does not activate cyclin D1 expression, but it acts cooperatively with STAT3 to induce cyclin D1 expression. Moreover, EGF treatment in GATA-4- or GATA-4/STAT3-expressing cells also led to cyclin D1 expression. These results may be due to EGF over-expression because GATA-4/STAT3 can synergistically activate EGF transcription. Consistent with this hypothesis, EGF treatment also led to the same result. In support of the relevance of EGF over-activation and cyclin D1 expression during cardiac differentiation described above, we assessed cyclin D1 expression in P19CL6 cells treated with different doses of EGF for various treatment durations. As shown in Figure S3, only the administration of 100 ng/ml EGF for 4 and 12 days resulted in enhanced cyclin D1 expression, and MHC expression was reduced or blocked in these cells (Fig. 3c). In agreement with this finding, enhanced cyclin D1 expression may partially account for the inhibition of cardiac differentiation, which is consistent with earlier studies indicating that enhanced cyclin D1 expression inhibits the differentiation of cardiomyocytes [29]. Finally, these results demonstrate a critical role of EGF in the control of cardiac differentiation.

The heart is derived from the mesoderm, under the precise regulatory hierarchies, mesodermal progenitor cells subsequently developed into cardiac progenitors, cardioblasts, and fully differentiated cardiomyocytes. In this process, transcription factors Islet 1 and MEF2C were subsequently expressed at the development stage of cardiac progenitors and cardioblasts, respectively, and cardiac myosin heavy chains was expressed in fully differentiated cardiomyocytes [21]. To determine the expression of Islet 1 and MEF2C during the cardiac differentiation of P19CL6 cells, we examined the expression level of Islet 1 and MEF2C protein in control P19CL6 cells, P19CL6 cells transfected with different expression vectors, and P19CL6 cells treated with different doses and different treatment periods of EGF. As shown in Figure S4 and S5, consistent with the results about MHC expression, the expression of Islet 1 and MEF2C proteins is inhibited when the expression of MHC is inhibited, which further demonstrated the critical roles of GATA-4 and EGF in the control of cardiac differentiation.

Discussion

As a well-regulated process essential for heart development, cardiac differentiation has been intensively studied

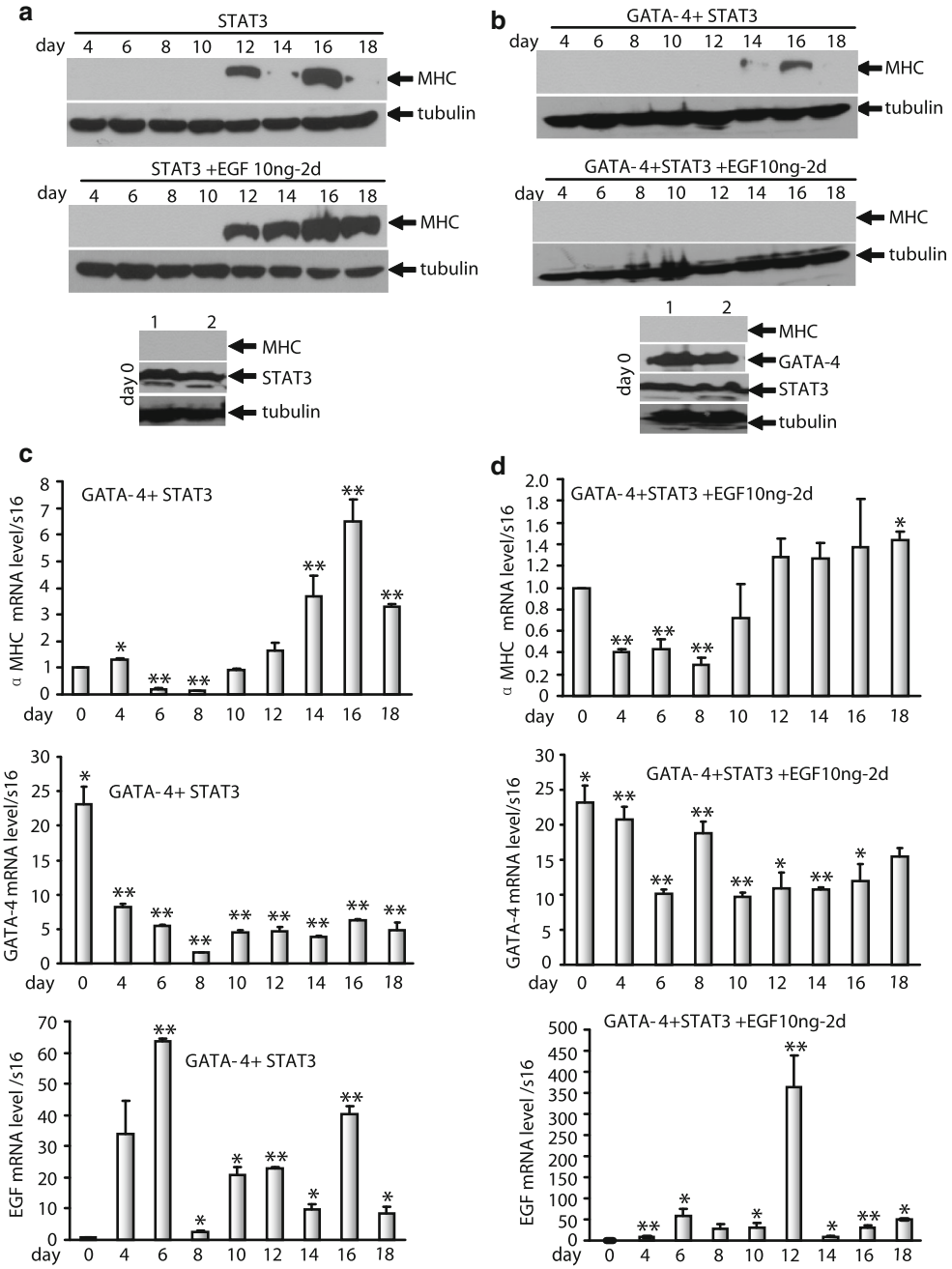


Fig. 7 EGF reversed STAT3-GATA-4 protein complex-induced cardiac myogenesis. **a** Stably expressed STAT3 induced the expression of sarcomeric MHC protein, compared with the DMSO vehicle control (Fig. 3a), which did not result in detectable expression of sarcomeric MHC until day 16 (*upper left*). Furthermore, the induction of sarcomeric MHC protein in P19CL6 cells stably expressing STAT3 is further enhanced by exposure to 10 ng/ml EGF for 2 days (*middle*). The cell lysates of the cells described above on day 0 were also subjected to immunoblot analysis (*lower*, *lane 1* STAT3, *lane 2* STAT3 + EGF 10 ng-2d). **b** Stably expressed STAT3 and GATA-4 induced the expression of sarcomeric MHC protein, compared to the DMSO vehicle control (Fig. 3a) (*upper left*), but the induction of sarcomeric MHC protein in P19CL6 cells stably expressing STAT3 and GATA-4 was blocked by exposure to 10 ng/ml EGF for 2 days (*middle*). Cell lysates described above on day 0 were also subjected to immunoblot analysis (*lower*, *lane 1* GATA-4 + STAT3, *lane 2* GATA-4 + STAT3 + EGF 10 ng-2d). **c**, **d** The mRNA levels of EGF and the cardiac-specific markers α-MHC and GATA-4 were determined in STAT3/GATA-4 stably expressing P19CL6 cells (**c**) and after 2 days of 10 ng/ml EGF treatment (**d**). The results are the mean ± SEM. $^{*}p < 0.05$ vs. control, and $^{**}p < 0.01$ vs. control

[14, 30]. Recently, GATA-4 was found to be the key modulator of cardiac differentiation [3, 15]. However, the mechanism by which GATA-4 achieves its roles is mostly unknown. In this study, using P19CL6 cells as models for cardiac differentiation, we determined that EGF is essential for cardiogenesis and is functionally linked to GATA-4 in inducing the cardiac differentiation of P19CL6 cells. Furthermore, EGF, a novel target of GATA-4, also regulates cardiac differentiation by modulating the cell cycle regulatory protein cyclin D1. More importantly, GATA-4 and STAT3, a downstream mediator of EGF signaling, function to recruit each other to the EGF promoter independent of EGF exposure. Interestingly, this interaction results in cooperative activation of EGF transcription but not in the promotion of cardiac differentiation, partly due to the activation of cyclin D1 expression by EGF. Our data indicate that EGF is a new target gene of GATA-4, and we provide mechanistic insight into the role of GATA-4 in cardiac differentiation.

GATA-4 is a member of the GATA transcription factor family, which contain zinc-finger domains [3], and plays a key role in heart development [3, 18]. Unlike its role in

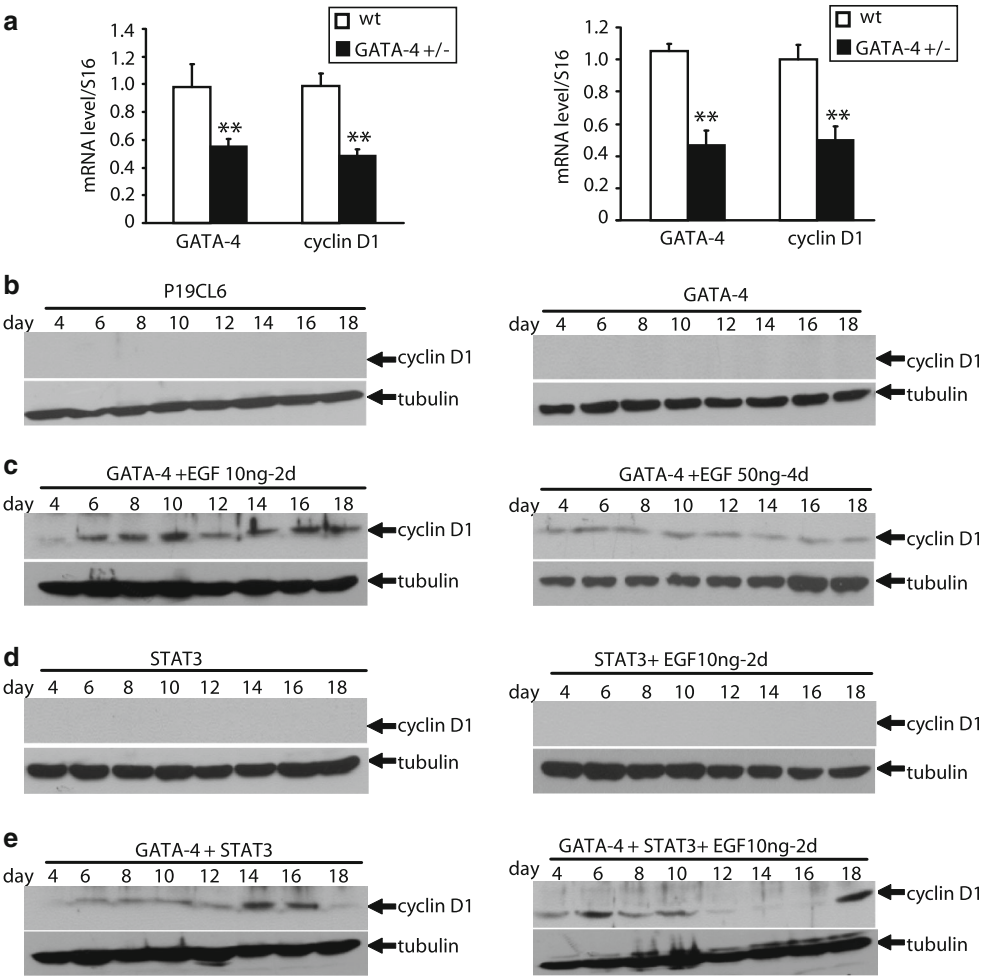


Fig. 8 The expression level of cyclin D1 during the differentiation of P19CL6 cells treated with EGF, untreated, or in cells stably transfected with different expression vectors. **a** The mRNA level of cyclin D1 is down-regulated in E12.5 (*left*) and E14.5 (*right*) ventricles of GATA-4+/− mice. RNA was extracted from the ventricles of GATA-4+/− mice and age-matched wild-type (wt) mice and the transcript levels for GATA-4 and cyclin D1 were determined. The results are the mean ± SEM. $^{**}p < 0.01$ vs. control. **b–e** The untreated cells, those treated with EGF, and those stably transfected with GATA-4 and/or the STAT3 expression vector were cultured for 18 days in 1 % DMSO. At the indicated time points, the cell lysates were subjected to immunoblot analysis with the cyclin D1 antibody

heart development, the discovery of GATA-4 as the key regulator in tumors was relatively recent [24, 32]. GATA-4 was found to be involved in colorectal and lung cancers and human adrenal tumors [24, 32]. However, the possible mechanisms through which GATA-4 is involved carcinogenesis are mostly unknown. Our present study provides compelling evidence that GATA-4 regulates EGF expression to affect cardiac differentiation. Because EGF plays important roles in inducing cancer cell epithelial–mesenchymal transition, proliferation, and invasion [8, 13, 31, 34], we speculate that GATA-4, a regulator of EGF during cardiac differentiation, may exhibit roles in carcinogenesis through the regulation of EGF expression. Because cancer cells grow uncontrollably, it would be interesting to determine whether and how GATA-4 regulates EGF expression to induce cancer cell differentiation into normal cells, analogous to its action during cardiac differentiation.

EGF was previously shown to promote a cardiomyoblastic phenotype and protect against low-flow ischemia-induced injury [11, 17, 26], indicating its role in the heart. Here, using immunoblot assays, we found that EGF induces the cardiac differentiation of P19CL6 cells in a concentration- and time-dependent manner. Unexpectedly, EGF treatment cannot reverse the cardiac differentiation block mediated by the depletion of GATA-4. Similarly, the enhancement of GATA-4 expression cannot rescue the inhibitory cardiac differentiation mediated by EGF RNAi. There may be two reasons for these findings. First, as shown in Figs. 1, S1A, and S1B, GATA-4 regulates EGF expression, and EGF also regulates GATA-4 expression, indicating that the regulation between GATA-4 and EGF may be bi-directional. Thus, the deletion of either gene may down-regulate the other, and restoring expression may not reverse the trend. Second, EGF signaling mediators may be involved in these processes. STAT3, a mediator of EGF signaling, controls GATA-4 expression and is essential for P19CL6 cell differentiation into cardiomyocytes [36]. Therefore, it would be interesting to examine whether STAT3 plays a role in the interplay between GATA-4 and EGF.

As revealed by chromatin immunoprecipitation (ChIP) assays, STAT3 was able to occupy the GATA consensus

sequence under basal conditions, and this binding is increased in response to EGF treatment. Interestingly, upon EGF stimulation, GATA-4 protein was also recruited to the STAT3 sites of the EGF promoter. We speculate that GATA-4, which is recruited to form the GATA-4/STAT3 complex, may move onto the EGF promoter as a feedback response to EGF signaling. Based on our co-immunoprecipitation and GST pull-down results, we found that the SH2 domain of STAT3 and the N-terminal TAD and the first zinc finger of GATA-4 are crucial for the interaction of these two proteins. Wang et al. showed that GATA-4 and STAT1, another member of the STAT family, interact with each other to cooperatively mediate the Angiotensin II responsiveness of the ANF promoter. Unlike STAT3, the N-terminal domain of STAT1 is essential for their association [43]. Therefore, it is possible that, in the heart, other members of the GATA and STAT families also associate with one another in response to signaling molecules and may be recruited onto specific target genes. In this regard, it would be interesting to determine whether interactions between STAT and GATA proteins are a general paradigm in response to cytokine and growth factor signaling.

The physical interaction between two proteins is always linked to a functional interaction. Therefore, the roles of the GATA-4/STAT3 complex during EGF activation were determined. Based on our transient transfection experiments, we found that GATA-4 and STAT3 cooperate synergistically to activate the EGF promoter, but the GATA-4/STAT3 protein complex cannot synergistically induce the cardiac differentiation of P19CL6 cells. Considering their synergy during the activation of EGF promoter, we speculate that over-activated EGF expression would not lead to further induction of cardiac differentiation. Four lines of evidence from our study are supportive of this speculation. First, EGF treatment fully blocked the induction mediated by the GATA-4/STAT3 complex. Second, when cells were treated with 10 or 50 ng/ml EGF for 2 or 4 days, GATA-4-induced cardiac differentiation is inhibited. Third, EGF induced cardiac differentiation in a dose- and time-dependent manner. A high concentration and prolonged EGF treatment caused the inhibition of cardiac differentiation. Fourth, EGF expression in the P19CL6 cells described above was significantly increased during differentiation. It is also of note that activation of cyclin D1 in these P19CL6 cells may partially contribute to the inhibition of cardiac differentiation. However, the precise molecular mechanisms of this observation are currently unknown. Accordingly, further studies will be undertaken to further investigate how other factors might also contribute to the inhibition of cardiac differentiation. Although the relevant mechanism is not fully understood, it is conceivable that the regulatory cascade of GATA-4/EGF/cyclin D1 could be a strong target for cardiac regeneration.

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