

***GATA-4* promotes the differentiation of P19 cells into cardiac myocytes**

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Abstract. The aim of this study was to investigate the effects of *GATA-4* on the differentiation of P19 cells into cardiomyocytes and to examine the relationship between *GATA-4* and cardiomyocytes. We constructed vectors to overexpress and silence *GATA-4*. These vectors, as well as empty ones were transfected into P19 cells. Subsequently, reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis were performed. The morphology of P19 cells during differentiation was observed using an inverted microscope. Total RNA was extracted from P19 cells. We used real-time PCR to evaluate the expression levels of 6 genes: *GATA-4*, *GATA-6*, transthyretin (*TTR*), α -fetoprotein (*AFP*), *Nkx2.5*, and α -myosin heavy chain (*α -MHC*). The gene expression pattern of these 6 genes is graphically shown for each group. The *GATA-4* mRNA level in cells overexpressing *GATA-4* was notably higher than that in the controls, whereas the levels in the controls were notably higher than those in the *GATA-4*-silenced P19 cells. The cell lines overexpressing *GATA-4* expressed higher levels of *Nkx2.5* and *α -MHC* than the controls. However, the controls expressed higher levels of *AFP*, *GATA-6* and *TTR* than the cells overexpressing *GATA-4*. The RNAi group expressed lower levels of *TTR*, *Nkx2.5*, and *α -MHC* than the controls, but there were no differences in the RNAi group and the controls with regard to the expression levels of *AFP* and *GATA-6*. The gene expression pattern in the cells over-

expressing *GATA-4* was biased toward the *Nkx2.5* and *α -MHC*. On the other hand, the gene expression pattern in *GATA-4*-silenced cells and the controls was biased toward the *TTR* and *AFP*. The overexpression of *GATA-4* enhances the differentiation of P19 cells into cardiac myocytes, whereas its down-regulation suppresses this trend.

Introduction

In higher vertebrates, cardiogenesis is a complex process that begins during the early stages of embryogenesis and involves the commitment of anterior lateral plate mesoderm cells to the cardiogenic lineage. These committed precursors subsequently differentiate into cardiomyocytes, whose proliferation is mainly responsible for heart development (1). The delicate balance between cell differentiation, proliferation, and apoptosis is crucial for proper organogenesis. Understanding the transcriptional and signaling programs that specify the cardiomyocyte fate from uncommitted progenitors, may provide important clues on the impact of cellular strategies for treating failing or infarcted heart tissue. *GATA* factors are comprised of a small family of highly conserved zinc finger transcription factors that play critical roles in the development of the cardiovascular, hematopoietic, digestive, and reproductive systems (2). *GATA-1*, *GATA-2*, and *GATA-3* are preferentially expressed in hematopoietic cells and regulate the proliferation and differentiation of blood cells during hematopoiesis (3). *GATA-4*, *GATA-5*, and *GATA-6* are predominantly expressed during cardiogenesis, in addition to other sites. The DNA-binding domains of *GATA-4*, *GATA-5*, and *GATA-6* are approximately 85% similar at the protein level. During early cardiac development, *GATA-4* expression is confined to the cardiac crescent, whereas later, transcripts are detected throughout the myocardium and endocardium (4,5). The null mutation of *GATA-4* results in the abnormal ventral folding of the embryo, failure to form a single ventral heart tube, and lethality by embryonic day (E) 10.5 (6,7). In humans, mutations in *GATA-4* result in congenital cardiomyopathies, including valve and septal defects (8,9). Both conditional knockout mice lacking *GATA-4* in specific cardiomyocytes, as well as transgenic mice expressing only 30% of the normal levels of *GATA-4* in the heart, display atrioventricular

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canal defects and a hypoplastic ventricular myocardium. Ventricular hypoplasia is thought to be associated with defects in cardiac morphogenesis and cardiomyocyte proliferation (10,11). However, the genetic programs under *GATA-4* control are not well understood. P19 cells, which are isolated from an experimental embryo-derived teratocarcinoma in mice, are multipotent and can differentiate into the cell types of all 3 germ layers. Due to their ability to form cardiomyocytes, P19 cells have been used to understand the role of cardiac-specific transcription factors and upstream signaling pathways in cardiac cell differentiation (12). We constructed vectors that overexpressed *GATA-4* and silenced RNA interference. We investigated the effects of *GATA-4* on myocardial cell differentiation by evaluating the expression levels of *GATA-4*, *GATA-6*, transthyretin (*TTR*), α -fetoprotein (*AFP*), *Nkx2.5* and α -myosin heavy chain (α -*MHC*) and thus created a chart of the expression patterns of these 6 genes.

Materials and methods

P19 cell culture and the induction of differentiation. P19 cells were cultivated as aggregates from days 0 to 6 in the presence of 1% α -minimal essential medium containing 10% fetal bovine serum and 1% Me₂SO (DMSO) in bacteriological dishes in a 5% CO₂ atmosphere at 37°C. The medium was replaced every 2 days thereafter. Some cell aggregates were harvested on day 5 of differentiation. On day 6, the other cell aggregates were transferred to cell culture flasks. The morphological changes in the P19 cells were examined under an inverted microscope (Nikon, Japan) equipped with phase-contrast objectives and a digital camera (Nikon).

Establishment of *GATA-4*-expressing transformants. The coding sequence of *GATA-4* was subcloned into the *KpnI* and *XbaI* sites of a pcDNA 3.1/myc-His A expression vector by using the oligonucleotides 5'-AGGGGTACCCGCGGTGATTATGTCCCATGACTGTCA-3' and 5'-GCTCTAGACCATGTACCAAAGCCTGGCCATGGCCGCCA-3' to generate a plasmid expressing the *GATA-4*-6X-His fusion protein. Expression vectors carrying the *GATA-4* coding sequence as well as empty ones, were transfected into P19 cells. Two days after transfection, neomycin (G418) was added to the medium (600 μ g/ml) in order to select transfected cells. Drug-resistant cells began to form small colonies 2 weeks after the addition of G418. Individual colonies were isolated and propagated, and the *GATA-4*-6X-His fusion protein was identified by Western blotting with an anti-*GATA-4*-6X-His antibody (Genetex, USA). The colonies expressing the highest levels of *GATA-4* were selected for cell differentiation studies. The resulting plasmids were confirmed by sequencing.

Construction of *GATA-4* short interference RNA expression plasmids. Three conserved cDNA fragments within the coding region of mouse *GATA-4* were identified using the basic local alignment homology search tool. Three pairs of reverse complementary oligonucleotides were designed and synthesized, and each pair contained a loop sequence (5'-TTCAAGAGA-3'), an RNA Pol III terminator (5'-TTTTTT-3'), and 5' single-stranded overhangs for ligation into *BbsI*- and

*Bam*HI-digested pGPU6/GFP/Neo vectors. These oligonucleotides were annealed and inserted into the short interference RNA (siRNA) expression vectors. The sequences of the cDNA fragments (sense strands) were i) 5'-GCCTCTACATGAAGCTCCATG-3', ii) 5'-GCTCCATGTCCCAGACATTCA-3', and iii) 5'-GCTATGCATCTCCTGTCACTC-3'. These fragments were used to create the vectors pGPU6-shGATA4-1491, pGPU6-shGATA4-1743 and pGPU6-shGATA4-1830, respectively. An insert-free pGPU6 vector served as the negative control. An insert control designed to target the *GAPDH* mRNA was used as the positive control (Ambion).

RNA interference. Five pGPU6/GFP/Neo vectors, including a negative and a positive control, were transfected into P19 cells, which were then grown in 6-well culture plates containing complete culture medium. A lipopolysaccharide-free vector (2 mg/well) was used for each transfection. At 48 h after transfection, cells and supernatants were collected for real-time polymerase chain reaction (PCR) detection.

Western blotting. Total proteins were isolated from cultured cells, separated on a 7.5% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. These membranes were incubated with a mouse anti-*GATA-4*-6X His antibody (Genetex) and goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (Amersham, UK). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham).

Total RNA isolation and real-time qRT-PCR. Total RNA was isolated from cultured P19 cells using the TRIzol method (Invitrogen). Single-strand cDNA was synthesized as follows: The reverse transcription mixture contained 2 μ g total RNA, 2 μ l oligo d(T) primer, 4 μ l 5X MMLV buffer, 1 μ l dNTP mix (concentration, 10 mM), 0.5 μ l RNasin (40 U/ μ l), 1 μ l MMLV reverse transcriptase (200 U/ μ l; Promega, USA), and 11.5 μ l sterile H₂O in a total volume of 20 μ l. The reaction was carried out at 42°C for 1 h, followed by heat inactivation at 95°C for 10 min. For real-time PCR, 2 μ l cDNA (40 ng) were added to 23 μ l of a master mix containing 1 U SYBR-Green (Applied Biosystems, Foster City, CA, USA) and 300 nM reverse and forward primers. cDNA was amplified for 40 cycles with an Applied Biosystems 7300 real-time PCR system (Applied Biosystems). The primer sequences used are listed in Table I. The PCR product level was calculated from the threshold cycle, the amplification cycle at which the emission intensity of the product rises above a set threshold level.

Statistical analysis. The results are expressed as mean \pm SD except that the results in Table II are expressed as mean. Data were analyzed using one-way analysis of variance and t- or t'-tests making corrections for multiple comparisons, as appropriate.

Results

Stable cell line overexpressing *GATA-4*. The P19 cell line which stably transfected with a *GATA-4* plasmid was

Table I. Primer sequences used.

Gene name	Product size (bp)	Reverse and forward primer (5'-3')	Ta (°C)
<i>GATA-4</i>	212	R: TGCTGTGCCCATAGTGAGATGAC F: GAAAACGGAAGCCCAAGAACC	61
<i>GATA-6</i>	302	R: CTCTTGGTAGCACCAGCTCA F: GCAATGCATGCGGTCTCTAC	55
<i>TTR</i>	254	R: GGCTGAGTCTCTCAATTC F: CTCACCACAGATGAGAAG	60
<i>AFP</i>	238	R: AGGCTTTTGCTTCACCAG F: TCGTATTCCAACAGGAGG	55
<i>Nkx2.5</i>	215	R: CCGGTCCTAGTGTGGA F: AGCAACTTCGTGAACCTTTG	57
<i>α-MHC</i>	143	R: CTTTCGCTCGTTGGGA F: ACCGTGGACTACAACAT	52
<i>GAPDH</i>	251	R: GCCAGTAGAGGCAGGGATGATGTTC F: CCATGTTCGTCATGGGTGTGAACCA	60

R, reverse primer; F, forward primer; Ta, annealing temperature.

Table II. Expression level of marker genes in 5-day-old EBs formed in different groups.

Expression region	Gene	Relative expression (ratio to maximum)			
		1	2	3	4
Endoderm and mesoderm	GATA-4/GAPDH	1.214E+00 (0.11)	9.157E-01 (0.09)	1.060E+01 (1)	8.173E-02 (0.01)
	GATA-6/GAPDH	4.343E+00 (1)	4.300E+00 (0.99)	2.346E+00 (0.54)	4.001E+00 (0.92)
Endoderm (hepatogenesis, visceral yolk sac)	TTR/GAPDH	2.160E+00 (1)	2.085E+00 (0.97)	1.409E+00 (0.65)	9.739E-01 (0.45)
	AFP/GAPDH	1.651E+00 (0.93)	1.774E+00 (1)	7.977E-01 (0.44)	1.546E+00 (0.87)
Mesoderm (cardiogenesis)	Nkx2.5/GAPDH	9.831E-01 (0.08)	1.024E+00 (0.08)	1.258E+01 (1)	3.870E-01 (0.03)
	α-MHC/GAPDH	9.361E-01 (0.10)	9.323E-01 (0.10)	9.464E+00 (1)	1.463E-01 (0.02)

1, blank group; 2, empty vector control group; 3, GATA-4-overexpressing group; 4, GATA-4-silencing group.

established and maintained in α-MEM containing 250 μg/ml G418. The GATA-4 protein expression was verified by Western blotting (Fig. 1), and the mRNA expression was verified by real-time RT-PCR (Fig. 2).

siRNA inhibition of GATA-4 expression. The P19 cell line which stably transfected with a GATA-4-silencing plasmid was established and maintained in α-MEM containing 250 μg/ml G418. Real-time PCR was used to identify *GATA-4* (Fig. 3). Compared with the *GATA-4* mRNA expression levels in the control group, those in the P19 cells that had been transfected with pGPU6-shGATA4-1491, pGPU6-

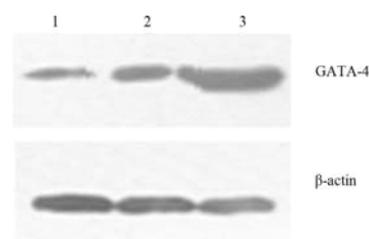


Figure 1. Expression of the *GATA-4* protein in P19 cells transfected with pcDNA3.1-GATA-4. Total proteins were isolated from stable cell lines and analyzed by Western blotting. Lane 1, normal P19 cell; Lane 2, pcDNA3.1; Lane 3, pcDNA3.1-GATA-4. The experiment was repeated 3 times, and the results were consistent.

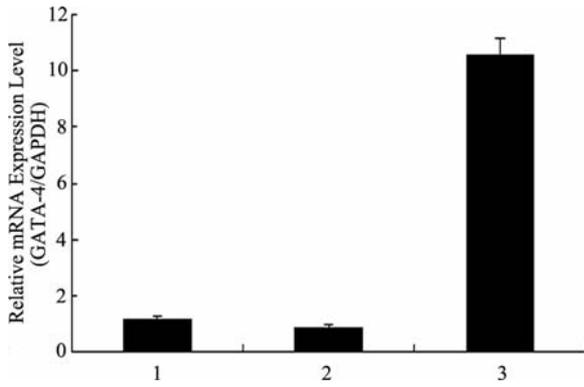


Figure 2. Expression of *GATA-4* mRNA in P19 cells transfected with pcDNA3.1-*GATA-4*. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, pcDNA3.1; Lane 3, pcDNA3.1-*GATA-4*. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

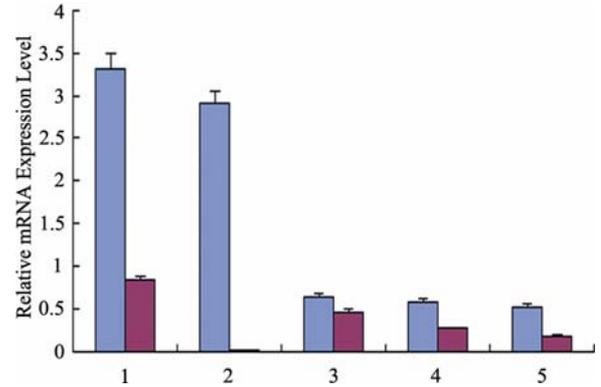


Figure 3. Expression of *GATA-4* and *GAPDH* mRNA in P19 cells transfected with pGPU6-sh*GATA-4* or pGPU6-sh*GAPDH*. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, pGPU6; Lane 2, pGPU6-sh*GAPDH*; Lane 3, pGPU6-sh*GATA4*-1491; Lane 4, pGPU6-sh*GATA4*-1743; Lane 5, pGPU6-sh*GATA4*-1830. * $p < 0.05$; $n = 4$.

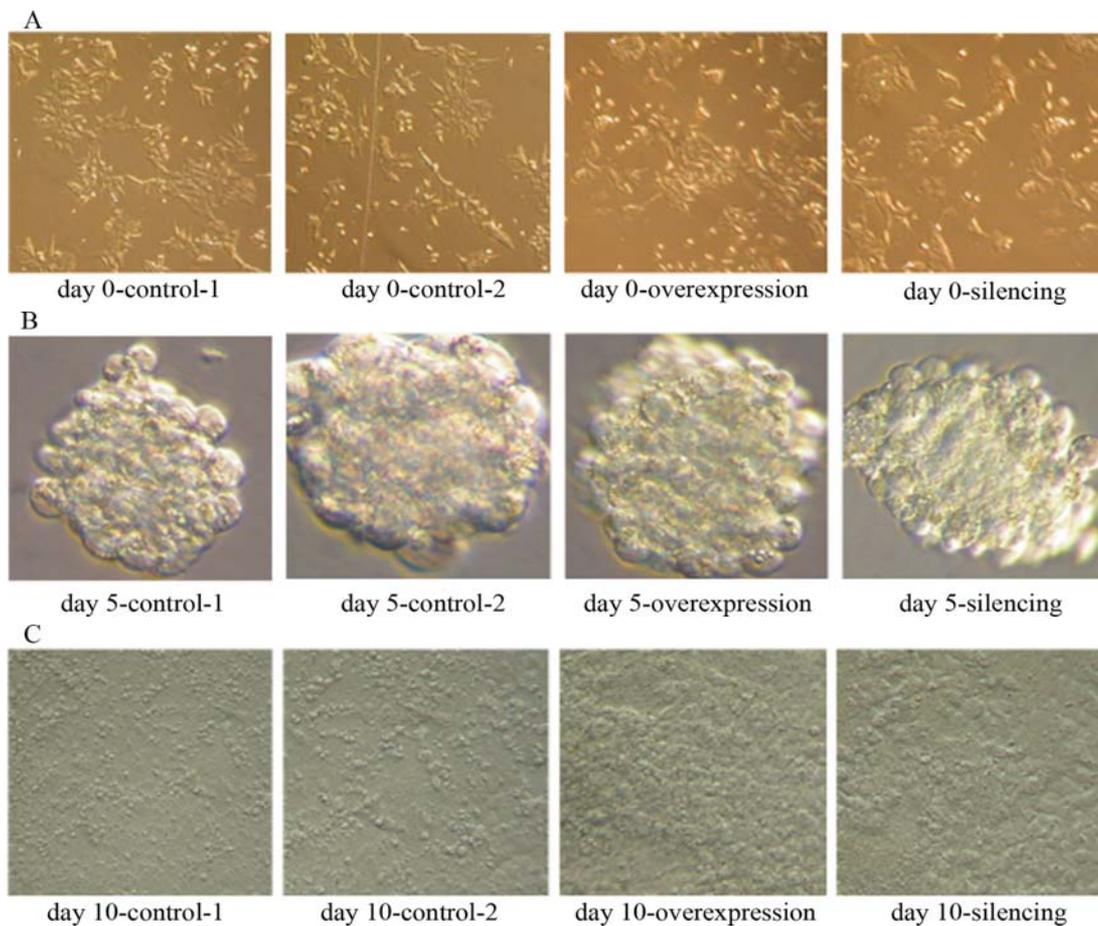


Figure 4. Morphology of P19 cells during differentiation. Control-1, normal P19 cells; control-2, pcDNA3.1; overexpression, pcDNA3.1-*GATA-4*; silencing, pGPU6-sh*GATA4*-1830.

sh*GATA4*-1743 and pGPU6-sh*GATA4*-1830 were 22.4, 20.4, and 18.5%, respectively.

Morphology of P19 cells during differentiation. P19 cells were derived from a pluripotent tumor in an early mouse embryo and were differentiated into various cell types depending on the environment (13). In order to investigate how P19 cells can be differentiated into cardiac cells, we

examined the morphological changes in the P19 cells. The morphology of P19 cell lines with *GATA-4* overexpressed or silenced on days 0, 5, and 10 of differentiation are shown (Fig. 4). The cardiac myocardial cells aggregated on day 4 of differentiation showed similar morphological changes to those observed during differentiation in all the groups. On day 10 of differentiation, the colonies of beating cells were observed more often in the overexpression group than in the

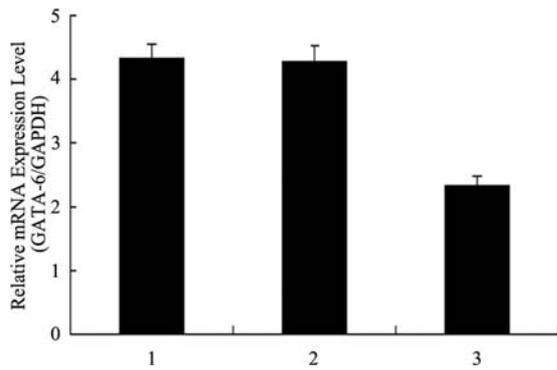


Figure 5. Expression of *GATA-6* mRNA in P19 cells transfected with pcDNA3.1-GATA-4. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cell; Lane 2, pcDNA3.1; Lane 3, pcDNA3.1-GATA-4. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

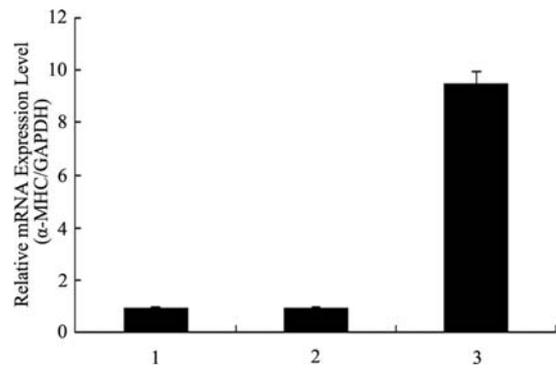


Figure 7. Expression of α -MHC mRNA in P19 cells transfected with pcDNA3.1-GATA-4. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, pcDNA3.1; Lane 3, pcDNA3.1-GATA-4. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

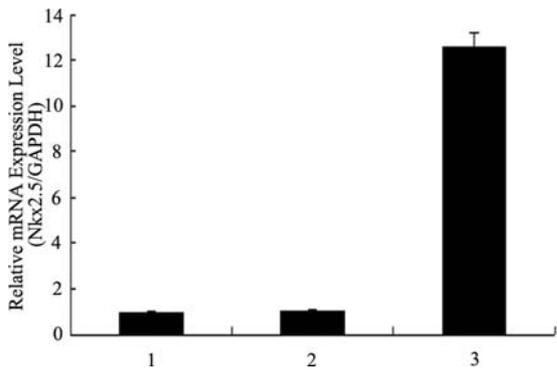


Figure 6. Expression of *Nkx2.5* mRNA in P19 cells transfected with pcDNA3.1-GATA-4. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, pcDNA3.1; Lane 3, pcDNA3.1-GATA-4. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

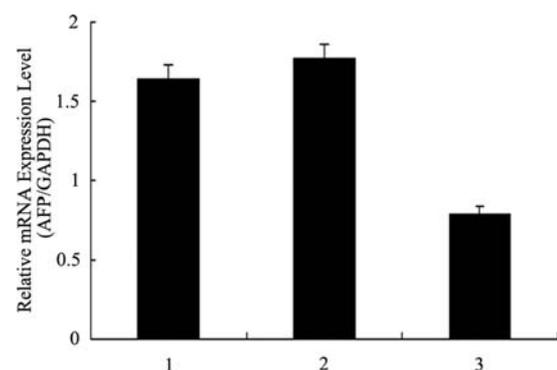


Figure 8. Expression of *AFP* mRNA in P19 cells transfected with pcDNA3.1-GATA-4. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, pcDNA3.1; Lane 3, pcDNA3.1-GATA-4. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

controls. However, they were hardly observed in the silenced group, indicating that *GATA-4* facilitates cardiac myocyte generation.

Expression levels of marker genes in 5-day-old embryoid bodies overexpressing *GATA-4*. The *GATA* transcription factors *GATA-4* and *GATA-6* are regarded as markers that are expressed in endoderm- and mesoderm-derived lineages, respectively, in embryoid bodies (EBs). *TTR* and *AFP* were used as endodermal markers that are specifically expressed in hepatocytes and the visceral endoderm of the yolk sac, respectively. The early cardiac transcription factor *Nkx2.5* and the cardiac muscle-specific α -MHC, were used as cardiac-specific markers (14). In this study, we used RT-PCR and analyzed the expression levels of the above-mentioned genes of 5-day-old EBs (the cells undergo a significant shift from an undifferentiated to a differentiated state during the culture of EB formation for 5 days) (14). We performed this analysis to evaluate the effects of *GATA-4* on the differentiation of P19 cells into cardiac myocytes and to understand the relationship between *GATA-4* and cardiomyocytes. The expression level of *GATA-6* was ~0.5 times lower in the

overexpression group than in the control group (Fig. 5), whereas the levels of *Nkx2.5* (Fig. 6) and α -MHC (Fig. 7) in the overexpression group were ~12.5 and 10 times lower, respectively, than the corresponding levels in the control group. The gene expression levels of *AFP* (Fig. 8) and *TTR* (Fig. 9) were, respectively, ~0.5 and 0.4 times lower than those in the control group.

Expression levels of marker genes in 5-day-old EBs in which the *GATA-4* gene is silenced. We used real-time PCR and examined the expression levels of *GATA-6*, *Nkx2.5*, α -MHC, *AFP*, and *TTR* in 5-day-old EBs to evaluate the effects of *GATA-4* on the differentiation of P19 cells into cardiac myocytes. There was no significant difference between the expression levels of *GATA-6* in the *GATA-4*-silenced cells and the control group (Fig. 10). In contrast, the levels of *Nkx2.5* (Fig. 11) and α -MHC (Fig. 12) were, respectively, ~0.6 and 0.8 times lower than the corresponding levels in the control group. There was no significant difference between the expression levels of *AFP* in the *GATA-4*-silenced cells and the control group (Fig. 13), whereas the *TTR* (Fig. 14) level was ~0.5 times lower than that in the control group.

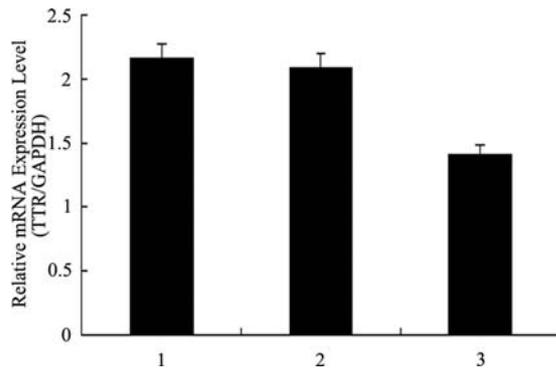


Figure 9. Expression of *TTR* mRNA in P19 cells transfected with pcDNA3.1-GATA-4. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, pcDNA3.1; Lane 3, pcDNA3.1-GATA-4. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

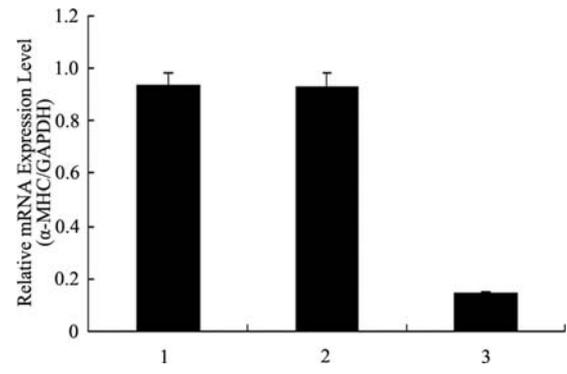


Figure 12. Expression of α -MHC mRNA in P19 cells transfected with pGPU6-shGATA4-1830. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, P19/pGPU6; Lane 3, P19/pGPU6-shGATA4-1830. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

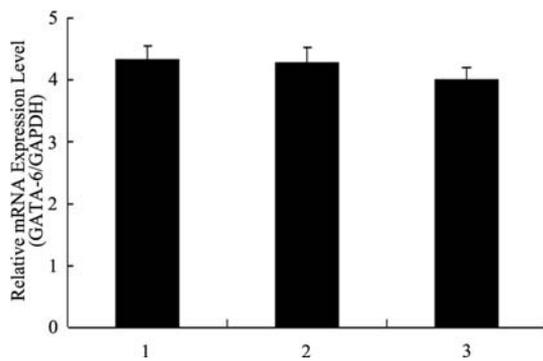


Figure 10. Expression of *GATA-6* mRNA in P19 cells transfected with pGPU6-shGATA4-1830. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, P19/pGPU6; Lane 3, P19/pGPU6-shGATA4-1830. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p > 0.05$; $n = 4$.

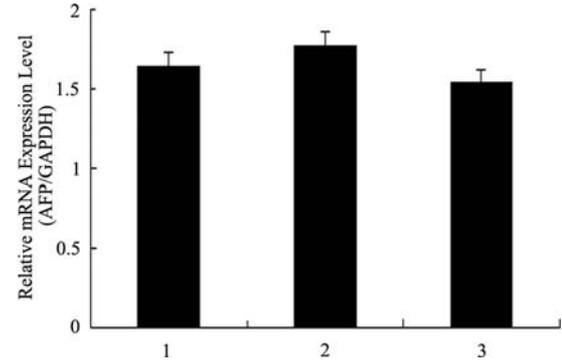


Figure 13. Expression of *AFP* mRNA in P19 cells transfected with pGPU6-shGATA4-1830. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, P19/pGPU6; Lane 3, P19/pGPU6-shGATA4-1830. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p > 0.05$; $n = 4$.

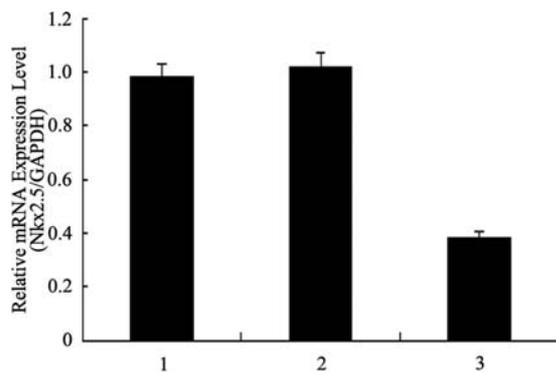


Figure 11. Expression of *Nkx2.5* mRNA in P19 cells transfected with pGPU6-shGATA4-1830. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, P19/pGPU6; Lane 3, P19/pGPU6-shGATA4-1830. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

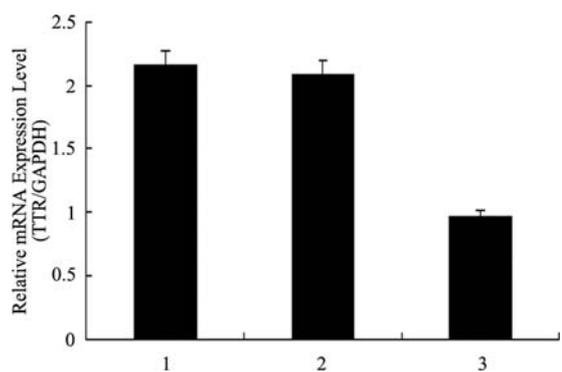


Figure 14. Expression of *TTR* mRNA in P19 cells transfected with pGPU6-shGATA4-1830. Total RNA was isolated from stable cell lines and analyzed by qRT-PCR. Lane 1, normal P19 cells; Lane 2, P19/pGPU6; Lane 3, P19/pGPU6-shGATA4-1830. Data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH expression. * $p < 0.05$; $n = 4$.

Characterization of the 5-day-old EBs in terms of gene expression. Table II shows the relative expression of the marker genes involved in the derivatives of the endoderm and mesoderm in 5-day-old EBs formed in different groups.

The characterization of the EBs in terms of relative gene expression was represented by the ratios to the highest level in each marker gene. The ratios of gene expression levels, shown within parentheses in Table II, were plotted on 6

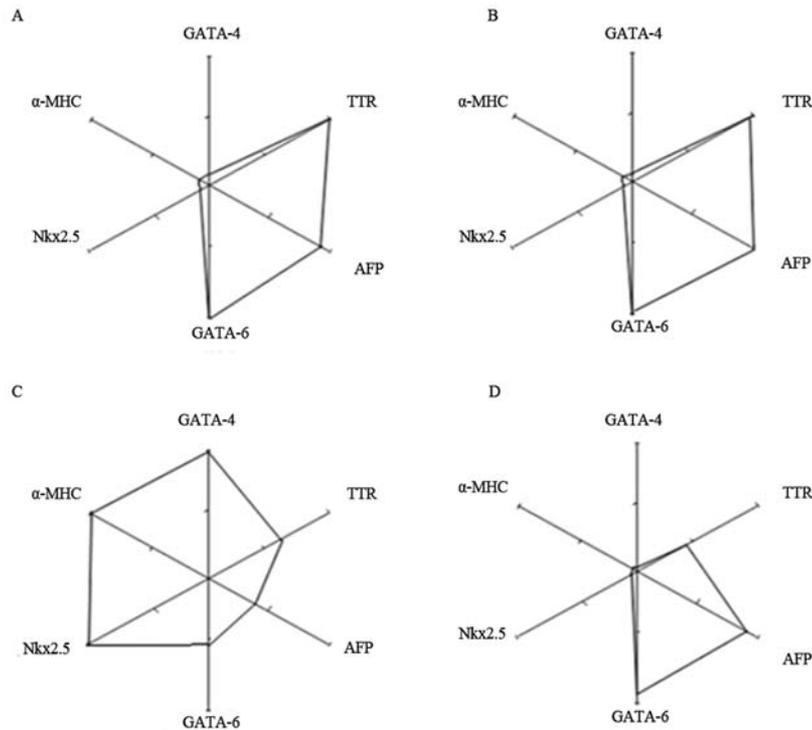


Figure 15. Charts of gene expression pattern in 5-day-old embryoid bodies formed in different groups. (A) Blank group; (B) empty vector control group; (C) GATA-4-overexpressing group; (D) GATA-4-silenced group. Original data are shown in Table II.

radial axes (Fig. 15). The ratios of the expression levels of *GATA-4* and *GATA-6* were plotted on the upper and lower vertical axes, respectively. The ratios of the expression levels of *Nkx2.5* and *α-MHC* were plotted on 2 axes on the left side, and the ratios of *TTR* and *AFP* were plotted on 2 axes on the right side. By connecting the plots on all the axes, an enclosed area appeared on the chart. This enclosed area defined the gene expression pattern that characterizes the differentiation status of the EBs.

The gene expression pattern in the EBs of the *GATA-4*-overexpressing group was biased toward the left side of the chart involving *Nkx2.5* and *α-MHC*. In contrast, the gene expression pattern in the EBs of the *GATA-4*-silenced group was similar to that in the control group. However, the enclosed area on the left side of the *GATA-4*-silenced group was smaller than the one on the right side, compared to the control group.

Discussion

Cardiogenesis involves a series of processes involving multiple cell line specialization, tubular structure formation, and precise assembly into the mature 4-chamber cardiac structure. This process entails a series of important events, including cell determination, migration, and differentiation. At the cellular level, it involves cell temporospatial differentiation, migration, proliferation and apoptosis. At the molecular level, a number of signal transduction pathways regulate the process of heart development (15,16). GATA transcription factors are zinc-finger DNA-binding proteins that regulate diverse pathways associated with embryonic morphogenesis and cellular differentiation (2). *GATA-4* was

initially described as an early cardiogenic marker, although the first requirement of mammalian *GATA-4* occurs within the extra-embryonic visceral endoderm for embryonic morphogenesis (6,17). On the basis of its expression in the early precardiac mesoderm and the phenotypes resulting from the various gain- and loss-of-function experiments, *GATA-4* is considered to be a key regulator of cardiogenesis (18). However, some other researchers believe that *GATA-4*-deficient embryonic stem cells retain the ability to differentiate into cardiomyocytes *in vitro*, and an analysis of cells from chimeric *GATA-4*-knockout mice has proven that *GATA-4* is not required for cardiomyocyte differentiation (19). Recent findings suggest that *GATA-4* can determine the fates of endoderms and facilitate, with temporal and spatial specificity, the generation of cardiomyocyte progenitors from the associated mesoderm (20). Further study is required in order to fully understand the relationship between *GATA-4* and cardiac myocytes.

DMSO is known to induce the differentiation of pluripotent P19 cells into embryonic myocardial cells. P19 cells are a useful model system for investigating the roles of various factors in cardiac induction and differentiation (12). P19 cells also represent an excellent model system for studying the regulation of myocardial electrophysiological differentiation at the molecular and functional levels (21). In the present study, the expression of *Nkx2.5* and *α-MHC* was up-regulated in the cell lines overexpressing *GATA-4* and down-regulated in those in which *GATA-4* was silenced. The early cardiac transcription factor *Nkx2.5* and the cardiac muscle-specific *α-MHC* are specific cardiac markers. Therefore, we believe that the overexpression of *GATA-4* enhances the differentiation of P19 cells into cardiac myo-

cytes and suppresses the differentiation of P19 cells into the cells generated from the endoderm.

Nkx2.5 is one of the early indicators of embryonic development (22). The transfection of *Nkx2.5* into zebrafish fibroblast cells can induce the expression of the markers of cardiac myocyte differentiation; this suggests that noncardiac cells overexpressing *Nkx2.5* can also be used to observe the process of heart differentiation (23). There is further evidence that *Nkx2.5* plays an important role in the differentiation of P19 cells into myocardial cells (24). In this study, the overexpression of *GATA-4* significantly increased the expression of *Nkx2.5* and therefore, we believe that *GATA-4* promotes the differentiation of P19 cells into cardiac myocytes. α -MHC (increased by the overexpression of *GATA-4* in this study) has been confirmed to be mainly expressed as a downstream target of *GATA-4* in the myocardium (25).

This study also showed that the overexpression of *GATA-4* downregulates the expression levels of *AFP*, *GATA-6* and *TTR*, suggesting that *GATA-4* can suppress the differentiation of P19 cells into the cells generated from the endoderm. The inhibition of *GATA-4* also downregulates the expression level of *TTR*. However, from the chart of gene expression patterns, the enclosed area on the left side of the *GATA-4*-silenced group was smaller than the one on the right side, compared to the control group. Thus, we believe that the inhibition of *GATA-4* suppresses the differentiation of P19 cells into cardiac myocytes. Consistent with the various gain- and loss-of-function experiments, *GATA-4* is considered a key regulator of cardiogenesis.

In conclusion, we believe that overexpression of *GATA-4* enhances the trend of P19 cell differentiation into cardiac myocytes, whereas the downregulation of *GATA-4* suppresses this trend. Further study of the function of *GATA-4* and the analysis of the relationship between *GATA-4* and cardiogenesis, may help to clarify the pathophysiological mechanisms of congenital heart disease and aid in improving diagnosis as well as in developing novel therapeutic strategies.

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