GATA4 expression is primarily regulated via a miR-26b-dependent post-transcriptional mechanism during cardiac hypertrophy

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Aims	GATA4 is a transcription factor that is up-regulated during cardiac hypertrophy and plays a fundamental role in myocyte growth and survival. In this study, we investigate the transcriptional vs. post-transcriptional mechanisms that are involved in regulating GATA4 in the heart during neonatal and pressure overload-induced hypertrophic growth.
Methods and results	GATA4 protein is significantly higher during pressure overload-induced (2.9 ± 0.4-fold) and neonatal (6.8 ± 1-fold) hypertrophic growth vs. the normal adult mouse heart. Using RNA polymerase II immunoprecipitation combined with deep sequencing, we confirmed that active transcription of the *Gata4* gene remained unchanged during hypertrophy, whereas it was two-fold higher in the neonatal vs. adult heart, commensurate with the mRNA levels. These results suggested a post-transcriptional mode of regulation of its expression, which prompted the identification of a conserved sequence in its 3′-untranslated region that was responsible for reduced translation via miR-26b. Overexpression of miR-26b reduced GATA4-dependent transcription, endothelin-induced hypertrophy, and sensitized the cells to apoptotic insults. Additionally, miR-26b targeted phospholipase C-β1, which, in turn, inhibited miR-26b expression, creating a double-negative feedback loop. Accordingly, overexpression of miR-26b in the heart inhibited up-regulation of its targets and the development of hypertrophy. However, knockdown of miR-26b is not sufficient for inducing hypertrophy.
Conclusion	Down-regulation of miR-26b in the heart is required for the up-regulation of GATA4 and the induction of pressure-induced cardiac hypertrophy. The results also underscore the functional relevance of miRNAs in regulating gene expression during cardiac hypertrophy.
Keywords	miR-26 • GATA4 • Hypertrophy • Phospholipase C-β

1. Introduction

A global increase in total RNA and protein synthesis is a hallmark of hypertrophy that underlies the increase in size and mass of the myocytes.[1–3] Superimposed on this change is a selective increase or decrease in the expression of specific genes in a manner that partially recapitulates the foetal gene programme.[4] In the mouse model, some of these changes include up-regulation of α-skeletal actin (αSkAc), atrial natriuretic factor (ANF), and β-myosin heavy chain (βMyh) and down-regulation of αMyh. An increase in the transcription factor GATA4 is potentially responsible for the increase in

αSkAc and ANF.[5] GATA4 is abundant during the development of the foetal and neonatal hearts relative to the fully mature heart, where its protein and transcriptional activity are up-regulated upon induction of cardiac hypertrophy.[6,7]

Myocyte-specific deletion of GATA4, with up to 95% loss of the protein, does not compromise mice survival but does result in cardiac dysfunction during adulthood.[8] In this model, transverse aortic constriction (TAC) and exercise failed to induce cardiac hypertrophy, but exhibited precipitous failure and increased apoptosis. These results were challenged, though, by another report that showed that cardiac-specific deletion of GATA4 may indeed result

in embryonic lethality.[9] In contrast, overexpression of GATA4 in the heart was sufficient for inducing cardiac hypertrophy.[10] *In vivo*, endothelin-1 mediates the increase in GATA4 during pressure-overload hypertrophy.[6] In accordance, inositol trisphosphate (IP_3), which is a product of endothelin-1-activated PLCβ1, induces an increase in GATA4 expression in cardiac myocytes.[11] However, the mode of regulation of GATA4 expression remains unknown.

MicroRNAs (miRNAs) are ~20 ribonucleotide-long molecules that have the capacity to post-transcriptionally inhibit a mRNA's translation or induce its degradation by targeting its 3′-untranslated region (3′UTR).[12] Others and we have shown that the levels of miRNAs fluctuate during development and disease, which implicates them in the underlying changes in gene expression.[12] In support, the expression pattern of miRNAs during cardiac hypertrophy to a great extent recapitulates that of the neonatal heart,[13] which mirrors that of translated genes.[4] Our previous study shows that miR-26b is down-regulated during cardiac hypertrophy, although its relevant targets in this context remain unknown. In this study, we show that the increase in GATA4 protein during cardiac hypertrophy is mainly a result of an miR-26b-dependent post-transcriptional regulation, as active transcription of its gene remains unchanged. We also confirm that down-regulation of miR-26b is necessary but not sufficient for the development of cardiac hypertrophy.

2. Methods

2.1 Animals

The work was done in accordance with US National Institute of Health Guidelines for the Care and Use of Laboratory Animals (no. 85-23). All animal protocols were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School.

2.2 Constructs

The following recombinant DNA constructs were cloned into an adenoviral vector: the stem-loop precursor of mmu-miR-26b and the antimiR-26b eraser (see Supplementary material online, *Figure S1* for expression data), full-length GATA4, GATA4 gene mutants lacking the 3′UTR (GATA4△3′UTR), the miR-26b (GATA4△26), or the miR-208 (GATA4△208) target sites, hairpin-forming silencing oligonucleotides for GATA4 and PLCb1, a concatamer of miR-26b-predicted target sequence within the GATA4 3′UTR downstream of a luciferase gene, the 3′UTR of mouse PLCb1 and a mutant lacking the miR-26b target sequence, a GATA4 luciferase reporter, and a skeletal actin luciferase reporter (see Supplementary material online for more details). All the constructs described in this section were cloned, propagated, and titered, as previously described by Graham and Prevec.[14]

2.3 Chromatin immunoprecipitation-deep sequencing

C57/Bl mice were subjected to either a sham or a TAC operation. After 4 days, those hearts, in addition to a pool of 1-day-old neonatal mice heart, were analysed by chromatin immunoprecipitation using anti-polymerase II antibody followed by deep sequencing (Genpathway Inc.; see Supplementary material online for more details).

2.4 Culturing cardiac myocytes and infection with adenoviruses

Cardiac myocytes were prepared as described previously.[15] Briefly, 1- to 2-day-old Sprague−Dawley rats were euthanized by dislocation of the neck and the hearts immediately removed through an incision left of the sternum. After dissociation with collagenase, the cardiac myocytes were differentially separated by Percoll gradient centrifugation followed by a differential pre-plating step for further separation of non-cardiac cells. Myocytes were then plated in Dulbecco's modified essential medium/Ham F12 (1:1) supplemented with 10% foetal bovine serum (10% FBS). In this medium, myocytes were infected with Ad.miR-26b; however, they were infected with Ad.miR-26b-eraser in the absence of 10% FBS, as miR-26b levels increase under these conditions. See Supplementary material online for more details.

2.5 Northern blotting

Northern blotting was done as described previously.[16]

2.6 Western blotting

Ten micrograms of protein extract from cultured myocytes, or 25 μg from tissue, were analysed on 4−20% SDS−PAGE (Criterion gels; Bio-Rad). Information on the primary antibodies used is provided in the Supplementary material online. The signal was detected and quantified by the Odyssey Imaging System (LI-COR).

2.7 Immunocytochemistry

Myocytes were plated on gelatin-coated glass slides and treated as described in the figure legends. The cells were then fixed, immunolabelled, and mounted as detailed in the Supplementary material online.

2.8 Luciferase assay

Cultured neonatal myocytes were infected with luciferase reporter vectors under the conditions specified in each experiment. Protein was extracted after 24 h and the luciferase activity was measured using the Luciferase Reporter Gene Assay, high-sensitivity kit (Roche), and an L_{max} multiwell luminometer. The results were then normalized to protein concentration.

2.9 Measurement of IP_3

Myocytes were infected with Ad.miR-26b or a control virus for 24 h before stimulating them with 10% FBS or 100 nM ET-1 for 15 min. The cells were harvested in phosphate-buffered saline and the IP_3 measure by HitHunter™ Inositol (1,4,5)-Trisphosphate Assay Fluorescence Polarization Detection kit as recommended by the manufacturer (DiscoveRx; see Supplementary material online for more details).

2.10 Real-time quantitative PCR

Total RNA was extracted by TRIzol Reagent (Invitrogen). A two-step real-time PCR was performed using High Capacity cDNA Reverse Transcription Kit and TaqMan® Gene Expression Assay (Applied Biosystem) for the indicated genes (see Supplementary material online for more details). A two-step real-time PCR for microRNA was performed using TaqMan® MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assay for the indicated miRNA (Applied Biosystem).

2.11 Caspase assay

Caspase-3 activity was measured using ApoTarget Caspase-3 Protease Assay (Biosource, Invitrogen), as recommended by the manufacturer.

2.12 Construction of the miR-26b transgenic mouse

The miR-26b transgene was constructed using the stem-loop precursor of mmu-miR-26b, cloned downstream of the 5.5 kb αMyh promoter and upstream of an SV40 polyadenylation signal.

2.13 Echocardiography

Mice were anaesthetized with 2.5% avertin (0.010−0.015 mL/g body weight) administered by intraperitoneal injection. The adequacy of the

anaesthetic was confirmed by the loss of tongue retraction reflex. Trans-thoracic echocardiography (Sequoia C256; Acuson, Mountain View, CA, USA) was performed using a 13 MHz linear ultrasound transducer (see Supplementary material online for more details). This procedure was terminal, as the heart was harvested while the mouse was under the influence of the anaesthetic.

2.14 Haemodynamic measurements

Mice were terminally anaesthetized as described above, and a 1.4 Fr (Millar Instruments) catheter-tip micromanometer catheter is inserted through the right carotid artery into the aorta and then into the left ventricle (LV) where pressures, dp/dt, and $-dp/dt$ are recorded.

2.15 Transverse aortic constriction

Twelve-week-old mice are anaesthetized intraperitoneally with a mixture of ketamine (65 mg/kg), xylazine (13 mg/kg), and acepromazine (2 mg/kg). The adequacy of the anaesthetic is confirmed by the loss of tongue retraction reflex. The transverse thoracic aorta between the innominate artery and the left common carotid artery was dissected free and a 7-0 braided polyester suture was tied around the aorta against a 26 G needle with the aid of an operating microscope. The needle was removed, the chest closed, and the mice were extubated and allowed to recover in a Thermocare unit (temperature 88°F or 31°C; humidity 30−50%; oxygen 1−2 mL/min, low flow range). Post-operative buprenorphine (0.01−0.05 mg/kg) was administered subcutaneously every 12 h, as needed. Cardiac function was assessed after 2 weeks before the mice were sacrificed and the hearts harvested for analysis. See the Supplementary material online for more details.

2.16 Statistics

For comparing two experimental groups, we used an unpaired, two-tailed, Student t-test (Excel software) for calculating the probability values. A value of $P < 0.05$ was considered significant.

3. Results

3.1 GATA4 protein is up-regulated during cardiac hypertrophy, while active transcription of its gene remains unchanged

While previous reports show that the GATA4 protein is up-regulated during cardiac hypertrophy,[7] there has been no discernable increase in its mRNA levels (Gene Expression Omnibus database,[17] GEO accession GDS2258; Mirotsau et al., 2006 and GEO accession GDS794; Zho et al., 2004).[18,19] To determine the mode of regulation of GATA4 expression during hypertrophic growth of the heart, we contrasted the levels of its protein, mRNA, and RNA polymerase II (RNA pol II)-dependent transcription of its gene in the adult mouse heart after TAC and in the neonatal heart, relative to the adult heart. Western blot analysis shows that the protein levels were 2.9 ± 0.4-fold higher in the hypertrophied heart after 4 days of TAC (Figure 1A and B). Similarly, 1-day-old neonatal hearts, which are in the process of hypertrophic growth, exhibited 6.8 ± 1-fold higher GATA4 relative to the fully mature adult heart (Figure 1A and B). The cardiac ankyrin repeat domain protein 1 (Ankrd1/CARP) is shown here as a positive hypertrophy marker,[20] which increases 7 ± 2.6 folds during TAC-induced hypertrophy and is 2.7 ± 0.2-fold higher in the neonatal vs. adult heart. The results confirm the up-regulation of GATA4 during cardiac hypertrophic growth conditions and suggest a transcriptional and/or post-transcriptional mode of regulation.

To conclusively address the transcription status of the Gata4 gene, we performed chromatin immunoprecipitation with anti-RNA pol II followed by extensive sequencing [chromatin immunoprecipitation-deep sequencing (ChIP-Seq)] of the attached DNA from the TAC-induced, sham, and neonatal hearts. The sequencing data were aligned to the genome using Affymetrix' Integrated Genome Browser (IGB), which revealed the fragment density of bound RNA pol II (y-axis) along the chromosome coordinates (x-axis) (Figure 1C). The panels in Figure 1C show the results of this alignment with Gata4, Ankrd1 (Carp), and Gapdh genes (Figure 1C). The total fragment density within the gene boundary in the neonatal and TAC genes relative to the adult gene was normalized to those of GAPDH and is plotted in Figure 1D. The data show that active transcription of Gata4 is unchanged during TAC, but was two-fold higher in the neonatal vs. adult heart. This is in contrast to the Ankrd1, which increased 10.7-fold during TAC, consistent with cDNA microarray data referred to above. Thus, the data point to a post-transcriptional mechanism in regulating GATA4 levels during TAC.

Post-transcriptional regulation can be a function of mRNA stability or the efficiency of translation. To discern between these two possibilities, we measured GATA4 mRNA levels using quantitative PCR (qPCR). The results were equivalent to those of gene transcription for both GATA4 and CARP. In specific, there was no increase in GATA4 mRNA during TAC, compared with a 6.4 ± 2.37-fold increase in CARP mRNA, while the neonatal heart had a 1.6 ± 0.35- and 3 ± 0.45-fold higher GATA4 and CARP mRNA, respectively, than the adult heart (Figure 1E). Cultured isolated myocytes also exhibited an increase in GATA4 protein when stimulated with FBS, which was not associated with an increase in mRNA (Figure 1F and G). The data, therefore, indicate that the increase in GATA4 during TAC and in the neonatal heart may be a function of translational control.

3.2 GATA4 is a predicted target of miR-26a/b and its expression levels inversely correlate with it

We used the miRNA target prediction engine, TargetScan,[21] to identify putative miRNA-targeting sites in the 3′UTR of GATA4 mRNA (Figure 2A and B). We expected that the relative levels of an miRNA differentially expressed during development or pressure-induced hypertrophy would have an inverse expression pattern relative to its targets. miR-26a, miR-26b, miR-122, miR-499, miR-208, and miR-200bc were predicted to target GATA4 through a site broadly conserved among human, mouse, rat, dog, and chicken with a probability of conserved targeting of 0.67 for miR-26b (Figure 2B). Using northern blot analysis, we found that, in particular, miR-26b is higher is all the adult vs. neonatal organs, in contrast to its predicted targets, GATA4 and PLCβ1 proteins, which are generally higher in the neonatal tissue vs. the adult tissues (Figure 2C and D). Likewise, miR-26b was down-regulated in a time-dependent fashion, after serum stimulation of isolated myocytes (Figure 2E). The results suggested that miR-26b might target and depress GATA4 expression.

3.3 miR-26b directly targets and inhibits the expression of GATA4

To determine whether miR-26b directly targets and inhibits GATA4, we used several approaches. First, we examined whether
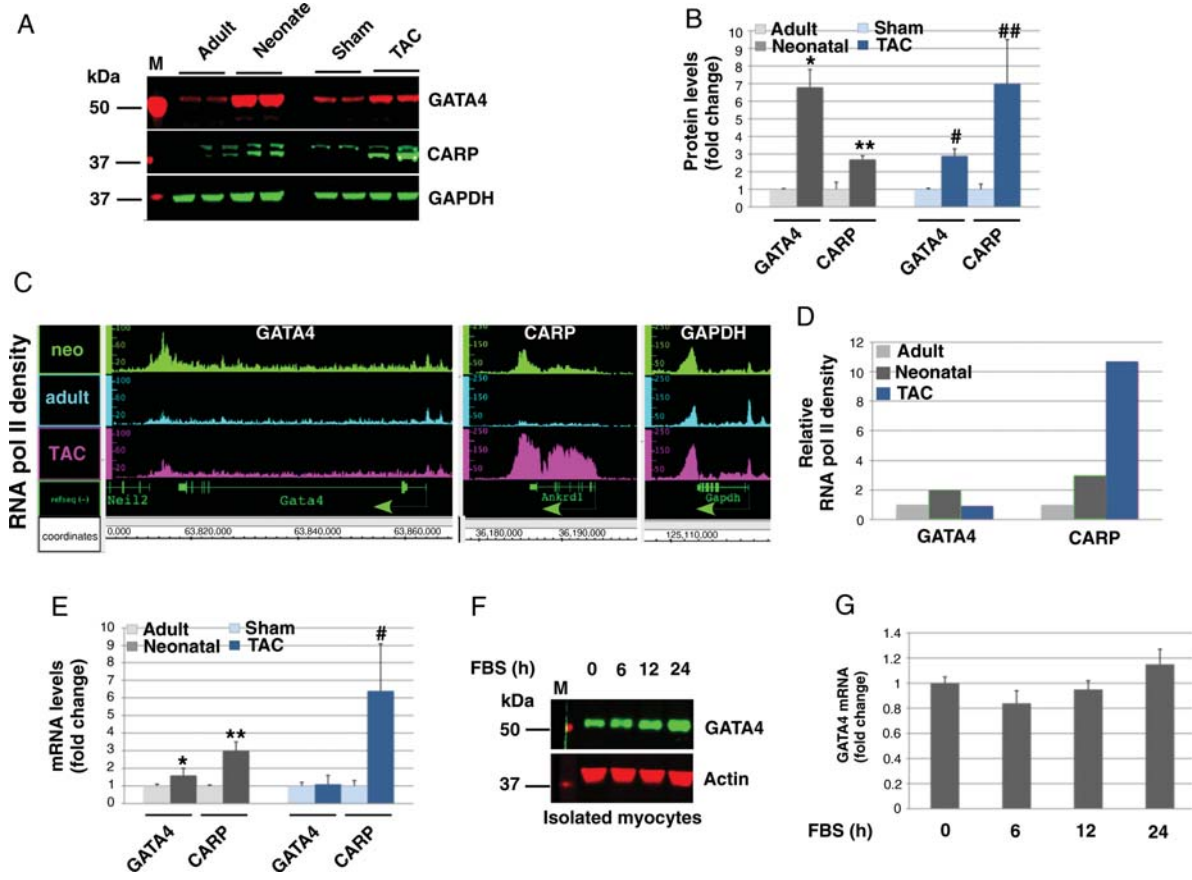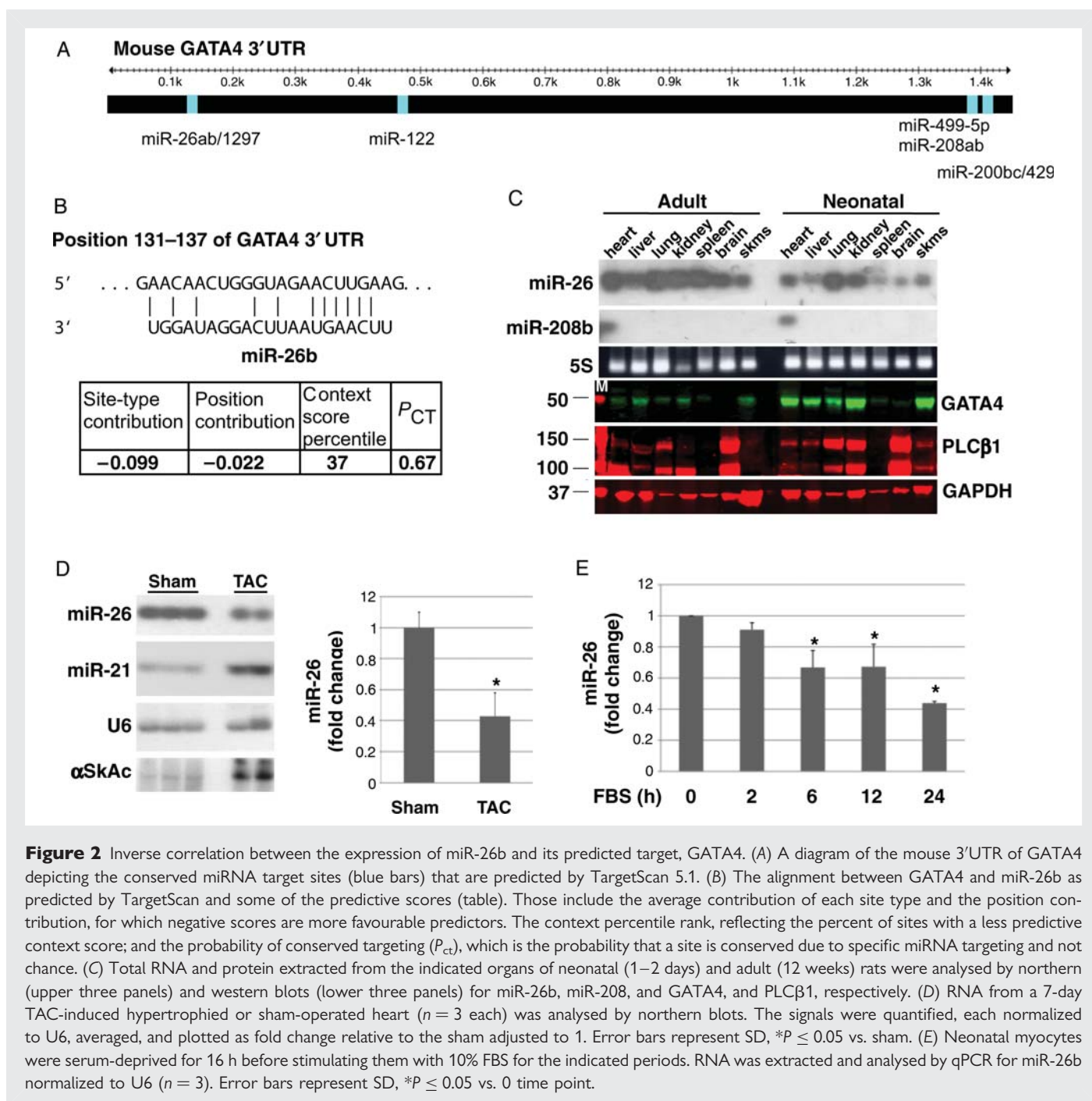
Figure 1 Transcriptional vs. post-transcriptional regulation of GATA4 during cardiac growth. (A−E) Protein, DNA, and RNA were extracted from the hearts of 2-day-old (neonatal), 12-week-old (adult), and 12-week-old subjected to a TAC or sham operation for 4 days, C57/BL mice ($n = 5$ each). (A) The protein was analysed by western blots. (B) The signals were quantified and normalized to GAPDH, and the results averaged and plotted as fold change in protein levels with the adult and sham levels adjusted to 1. The error bars represent SD, $*P \leq 0.05$ vs. adult GATA4, $**P \leq 0.005$ vs. adult CARP, $^{#}P \leq 0.05$ vs. sham GATA4, $^{##}P \leq 0.005$ vs. sham CARP. (C) Neonatal (green), pressure-induced hypertrophied (TAC, purple), and adult heart (blue) DNA were analysed by ChIP-Seq. Shown are IGB images for *Gata4*, *Ankrd1* (*Carp*), and *Gapdh* genes. The sites of bound RNA pol II are represented by vertical bars along the *y*-axis, the height of those bars along the *x*-axis represent the levels of bound RNA pol II. The genes' exons and introns in the form of a green line with superimposed bars (exons) are drawn to scale along the coordinates. (D) The total RNA pol II bound within the boundaries of each of the *Gata4* and *Carp* genes was normalized to *Gapdh* and plotted as relative values. (E) The RNA was analysed by qPCR, normalized to GAPDH, and plotted as fold change in mRNA levels with the adult and sham levels adjusted to 1. The error bars represent SD, $*P \leq 0.05$ vs. adult GATA4, $**P \leq 0.005$ vs. adult CARP, and $^{#}P \leq 0.05$ vs. sham GATA4. (F and G) Neonatal myocytes were serum-deprived for 16 h before stimulating them with 10% FBS for the indicated periods. Protein and RNA were extracted and analysed by western blots for GATA4 protein ($n = 2$) and qPCR for GATA4 mRNA ($n = 3$). The results were normalized to GAPDH, averaged, and plotted as fold change relative to the 0 time point adjusted to 1. Error bars represent SD.

overexpression of miR-26b via viral delivery in isolated cardiac myocytes inhibits the expression of endogenous GATA4 or a luciferase vector harbouring the miR-26b target sites within its 3′UTR. Western blot analysis shows that miR-26b very effectively inhibited the expression of endogenous GATA4 (>90%) and the miR-26b-targeted luciferase vector (~50%), but not GAPDH, Myh, or a control luciferase vector (*Figure 3A* and *B*). Conversely, an anti-sense miR-26b eraser modestly enhanced GATA4 expression (70%).

To assess the direct influence of miR-26b and miR-208 target sites within the 3′UTR on the expression of GATA4, we generated three deletion variants of its cDNA: one lacking the 3′UTR (GATA4△3′UTR), a second lacking the miR-26b site (GATA4△26), and a third lacking the miR-208 site (GATA4△208) (*Figure 3C*). The expression levels of these constructs were compared with the

full-length GATA4 cDNA in growth-arrested cardiac myocytes, which usually have ~2× higher levels of miR-26b than growth-stimulated cells. Western blot analysis reveal that deletion of the 3′UTR or the miR-26b target site within it, but not the miR-208 site, enhances the expression of GATA4 cDNA by a maximum of approximately two-fold (*Figure 3C* and *D*). These results confirmed that miR-26b is a negative regulator of GATA4 expression in neonatal cardiac myocytes via directly targeting a predicted target site within the 3′UTR.

Furthermore, miR-26b inhibited a GATA4-luciferase and a skeletal actin-luciferase reporter constructs by >90% (*Figure 3E* and *F*), equivalent to the decrease in GATA4 seen in *Figure 3A*. Conversely, the miR-26b eraser enhanced the expression of the GATA4-luciferase reporter, although it was not sufficient for enhancing the skeletal actin

Figure 2 Inverse correlation between the expression of miR-26b and its predicted target, GATA4. (*A*) A diagram of the mouse 3′UTR of GATA4 depicting the conserved miRNA target sites (blue bars) that are predicted by TargetScan 5.1. (*B*) The alignment between GATA4 and miR-26b as predicted by TargetScan and some of the predictive scores (table). Those include the average contribution of each site type and the position contribution, for which negative scores are more favourable predictors. The context percentile rank, reflecting the percent of sites with a less predictive context score; and the probability of conserved targeting (P_{ct}), which is the probability that a site is conserved due to specific miRNA targeting and not chance. (*C*) Total RNA and protein extracted from the indicated organs of neonatal (1–2 days) and adult (12 weeks) rats were analysed by northern (upper three panels) and western blots (lower three panels) for miR-26b, miR-208, and GATA4, and PLCβ1, respectively. (*D*) RNA from a 7-day TAC-induced hypertrophied or sham-operated heart ($n = 3$ each) was analysed by northern blots. The signals were quantified, each normalized to U6, averaged, and plotted as fold change relative to the sham adjusted to 1. Error bars represent SD, $*P \leq 0.05$ vs. sham. (*E*) Neonatal myocytes were serum-deprived for 16 h before stimulating them with 10% FBS for the indicated periods. RNA was extracted and analysed by qPCR for miR-26b normalized to U6 ($n = 3$). Error bars represent SD, $*P \leq 0.05$ vs. 0 time point.

promoter. The results prove that miR-26b inhibits GATA4-dependent transcription. In agreement, overexpression of miR-26b in cardiac myocytes inhibited endothelin- and phenylephrine-induced GATA4 expression (see Supplementary material online, *Figure S2* and *S3*) and endothelin-induced hypertrophy, in addition to sensitizing the myocytes to apoptosis (see Supplementary material online, *Figure S2*).

3.4 PLCβ1 regulates GATA4 expression via suppressing miR-26b, which directly targets and inhibits PLCβ1

IP$_3$, a product of endothelin-1-activated PLCβ1,[22] induces an increase in GATA4 expression in cardiac myocytes.[11] Using TargetScan, we

identified PLCβ1 as a, predicted, conserved target of miR-26b (see Supplementary material online, *Figure S1D*). Supplying the myocytes with exogenous miR-26b induced a dose-dependent down-regulation of PLCβ1 protein (*Figure 4A*) and luciferase activity produced by a luciferase-expressing vector that harbours the full-length 3′UTR of PLCβ1 (*Figure 4B*). In accordance, both ET-1- and FBS-induced IP$_3$ were significantly compromised in the presence of excess miR-26b as measured by fluorescence polarization detection (*Figure 4C*) and [^{3}H]IP$_3$ fractionation (see Supplementary material online, *Figure S4*). The data suggest that PLCβ1 is also a direct target of miR-26b.

Since IP$_3$ was previously reported to up-regulate the expression of GATA4, we questioned whether knockdown of PLCβ1 would reduce endogenous GATA4 via up-regulating miR-26b in cardiac myocytes.
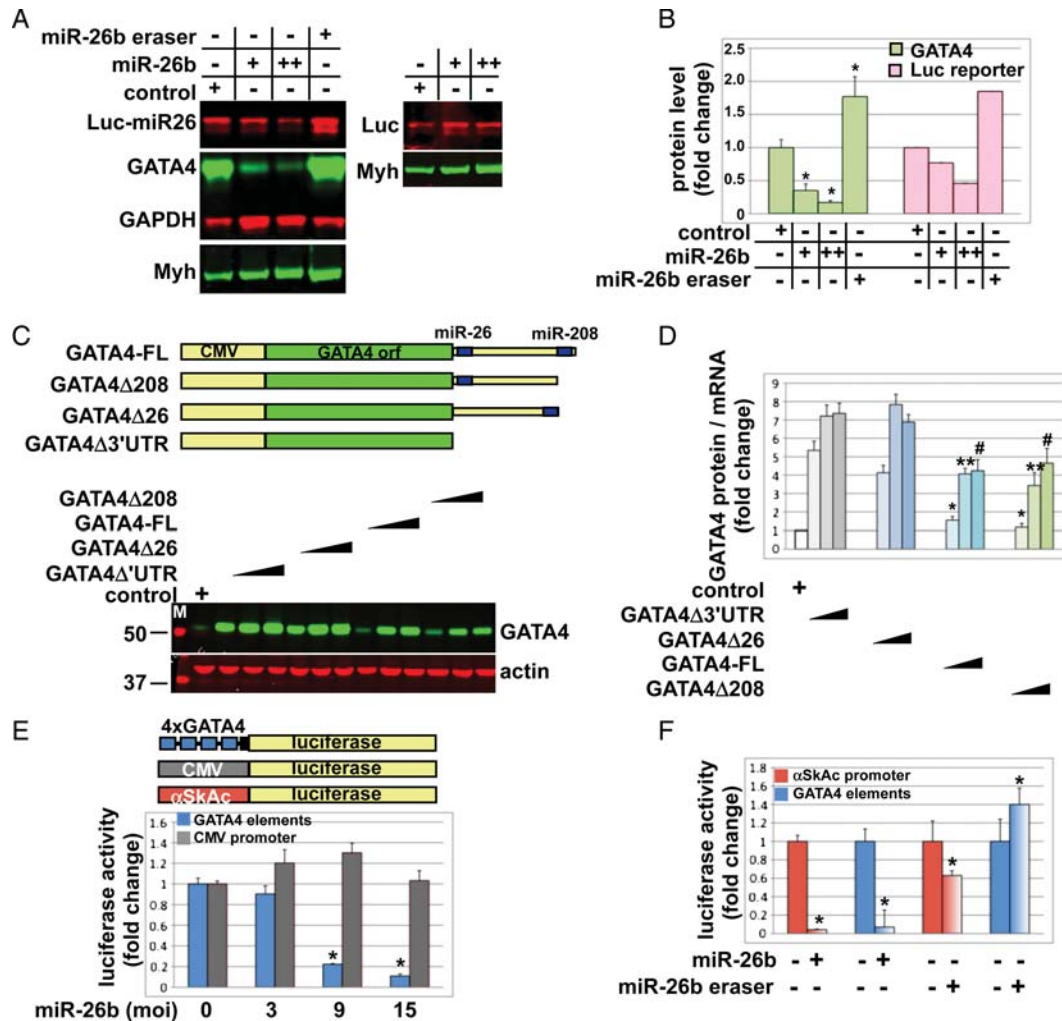
Figure 3 GATA4 expression is directly suppressed by miR-26b. (*A*) Neonatal cardiac myocytes were infected for 16 h with an adenovirus (Ad) expressing an miR-26b-responsive luciferase reporter gene (Luc-miR26). This was followed by infection with Ad.miR-26b precursor or Ad.miR-26b eraser for 24 h, as indicated (*n* = 4 each). The protein was extracted and analysed by western blots for luciferase and GATA4. A similar experiment was conducted with a control luciferase vector lacking the miR-26b target site (Luc) (*n* = 2). (*B*) The results in (*A*) were quantified, normalized to GAPDH, and plotted as fold change relative to the control condition adjusted to 1. Error bars represent SD, *$P \leq 0.005$ vs. control. (*C*) Ad vectors carrying full-length GATA4 (GATA4-FL), GATA4Δ208, GATA4Δ26, or GATA4Δ3′UTR were delivered to cultured myocytes in serum-deprived medium (*n* = 4 each). After 24 h, protein and RNA were extracted and analysed by western blots and qPCR. (*D*) GATA4 protein signal was quantified and normalized to mRNA levels to adjust for variation in viral doses or expression levels. The data were averaged and plotted as fold change relative to the endogenous GATA4 adjusted to 1. Error bars represent SEM, *$P < 0.001$ vs. lowest dose, **$P < 0.01$ vs. middle dose, #$P < 0.01$ vs. highest dose, of GATA4Δ3′UTR. (*E*) Ad.GATA4 or Ad.CMV luciferase reporters were delivered to cultured myocytes in the presence or absence of increasing doses of Ad.miR-26b (*n* = 3 each). After 24 h, luciferase activity was measured, normalized to total protein, averaged, and plotted as fold change in activity relative to the control level adjusted to 1. Error bars represent SEM, *$P \leq 0.005$ vs. control. (*F*) A similar experiment was conducted using an Ad.luciferase vector driven by the α-skeletal actin promoter (Δ394). The viruses were delivered to cultured myocytes in the presence or absence of Ad.miR-26b, Ad.miR-26b eraser, or a control. Error bars represent SEM, *$P \leq 0.005$ vs. control.

For that purpose, we supplied the cells with a short-hairpin RNA construct that would target PLCβ1 (PLCβ1-shRNA). This resulted in effective knockdown of the protein by $54 \pm 14\%$, but even more dramatically reduced the expression of GATA4 by >90% (*Figure 4D* and *E*), suggesting that the PLCβ1 plays a major role in positively regulating GATA4 expression. Since miR-26b has a similar dramatic effect on GATA4 expression (*Figure 3*), we hypothesized that PLCβ1's effect is mediated through up-regulation of miR-26b. Indeed, miR-26b levels were very sensitive to the knockdown of

PLCβ1 as it increased to a maximum of 50 ± 5 folds (*Figure 4F*). This effect was specific, as shRNA-PLCβ1 had no effect on miR-21 or miR-1 levels (data not shown). Conversely, neither shRNA targeting GATA4 or TF2B had any effect on miR-26b. However, we do not know the mechanism by which PLCβ regulates miR-26b. We, thus, predicted that ET-1 enhances the expression of both GATA4 and PLCβ1 via down-regulation of miR-26b. This is validated in *Figure 4G*, where ET-1-induced up-regulation of GATA4 and PLCβ1 was inhibited by overexpressing miR-26b (*Figure 4G*). The increase
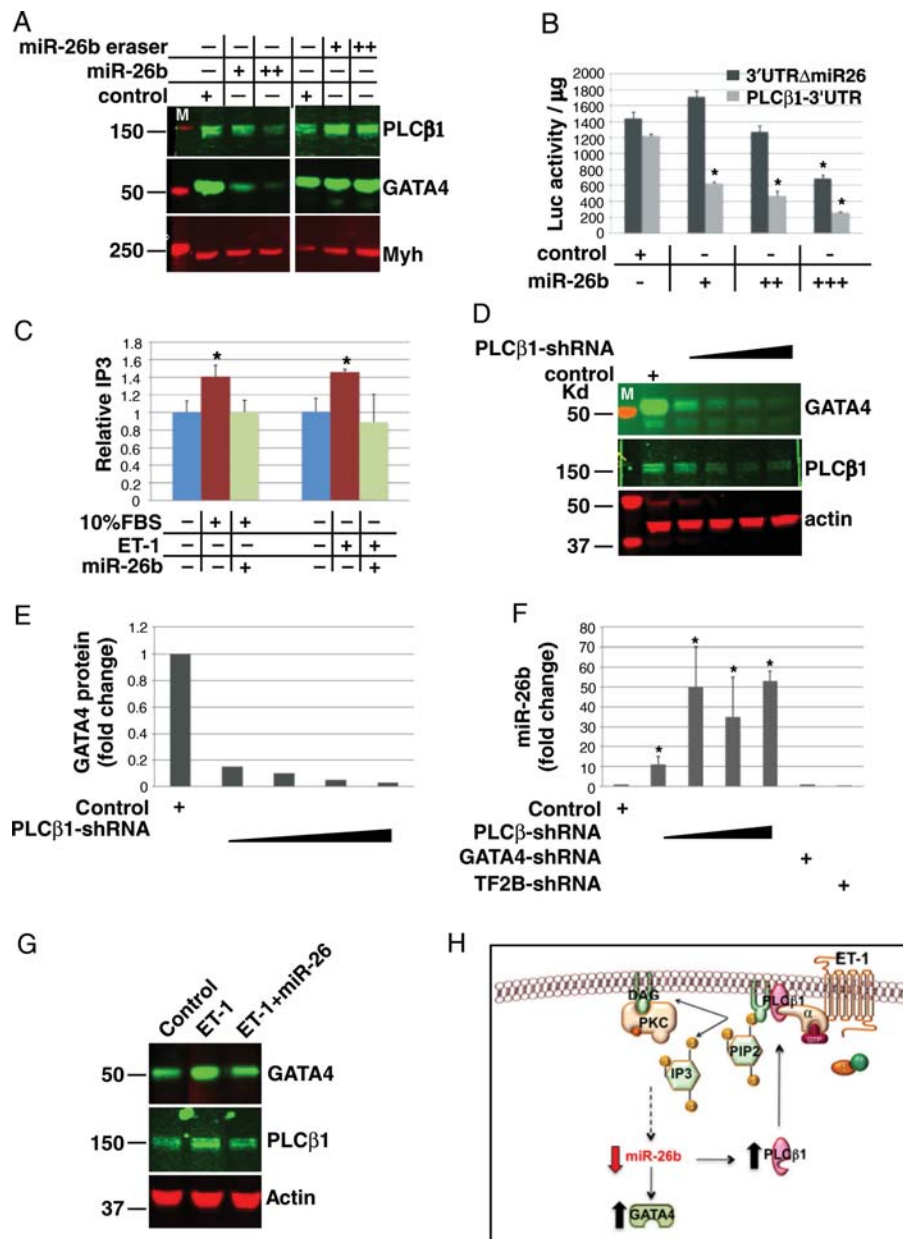
Figure 4 PLCβ1 is a negative regulator, and a target, of miR-26b. (*A*) Neonatal cardiac myocytes were infected with increasing doses of Ad.miR-26b or Ad.miR-26b eraser for 24 h, where indicated (*n* = 4 each). The protein was extracted and analysed by western blots. (*B*) PLCβ1-3′UTR or PLCβ1-3′UTRΔmiR26 luciferase reporters were delivered to cardiac myocytes in the presence or absence of increasing doses of Ad.miR-26b, as indicated. After 24 h, luciferase activity was measured, normalized to total protein content, averaged, and plotted as luciferase activity/μg protein. Error bars represent SEM, *$*P \leq 0.001$ vs. control. (*C*) Myocytes were infected with Ad.miR-26b or a control virus for 24 h before stimulating them with 10% FBS or 100 nM ET-1 for 15 min (*n* = 3 each). The cells were harvested in phosphate-buffered saline and the IP3 measured by a fluorescence polarization-dependent assay. The results were normalized to cell number, averaged, and plotted as relative quantities with basal levels adjusted to 1. Error bars represent SEM, *$*P \leq 0.01$. (*D*) Neonatal cardiac myocytes were infected with a control adenovirus or increasing doses of one expressing short-hairpin RNA-targeting PLCβ1 (PLCβ1-shRNA) for 48 h (*n* = 3). Protein was analysed by western blots. (*E*) The GATA4 protein signal was quantified and plotted as fold change relative to the control adjusted to 1. (*F*) Myocytes were treated as in (*D*), in addition to GATA4-shRNA- and TF2B-shRNA-expressing viruses (*n* = 3). RNA was analysed by qPCR. Results were normalized to U6, averaged, and plotted as fold change relative to the control adjusted to 1. (*G*) Cultured myocytes were supplemented with Ad.miR-26b maintained in serum-free medium for 24 h before stimulating them with 100 nM ET-1. After 24 h, protein was extracted and analysed by western blotting. (*H*) A depiction of how ET-1 regulates GATA4 expression. Stimulation of myocytes with ET-1 results in the activation of PLCβ1, which hydrolyses phosphatidyl inositol bis-phosphate (PIP$_2$) into diacylglycerol (DAG) and IP$_3$. This results in the down-regulation of miR-26b and, thus, up-regulation of its targets, GATA4 and PLCβ1, creating a feed-forward signalling loop.
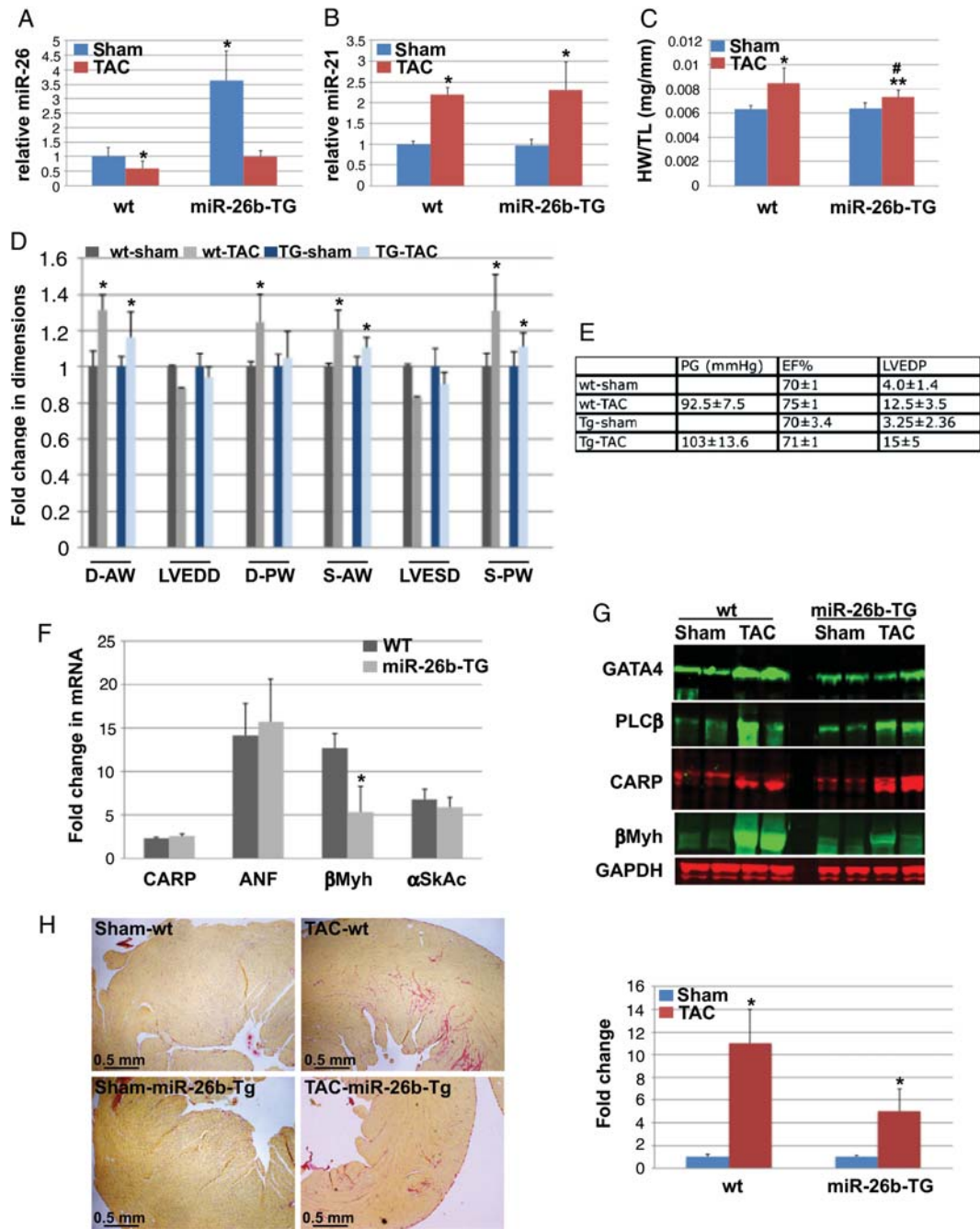
Figure 5 Normalization of miR-26b levels inhibits cardiac hypertrophy. (*A*–*G*) Twelve-week-old transgenic mice and gender- and age-matched wild-type (wt) FVB littermates were subjected to TAC or a sham operation (*n* = 6, each). Two weeks later, physical and functional cardiac parameters were assessed by echocardiography and haemodynamic measurements. This was followed by immediate isolation of the hearts from which RNA and protein were extracted. The RNA was analysed by qPCR for (*A*) miR-26b and (*B*) miR-21 for contrast. The results were normalized to GAPDH, averaged, and plotted as relative quantities relative to wt-sham adjusted to 1. Error bars represent SEM, *$P \leq 0.01$ vs. wt-sham. (*C*) The heart weights were normalized to tibial length, averaged, and plotted. Error bars represent SEM, *$P \leq 0.01$ vs. wt-sham, **$P \leq 0.05$ vs. Tg-sham, #$P \leq 0.01$ vs. wt-TAC. (*D*) Echocardiography measurements were averaged and plotted as fold change relative to sham-operated adjusted to 1. Those include D-AW (diastolic anterior wall width), LVEDD, D-PW (diastolic posterior wall width), S-AW (systolic anterior wall width), LVESD, and S-PW (systolic posterior wall width). (*E*) A table with the average values of the pressure gradients (PG) after TAC, % ejection fraction (EF%), and left ventricular end-diastolic pressure (LVEDP). (*F*) The RNA was analysed by qPCR for CARP, ANF, βMyh, and αSkAc. The results were normalized to GAPDH, averaged, and plotted as fold change relative to matched sham adjusted to 1. Error bars represent SEM, *$P \leq 0.01$ vs. sham. (*G*) The protein was analysed by western blots. (*H*) The hearts from wild-type and miR-26b-Tg mice, before and after sham or TAC surgeries, were fixed, sectioned, stained with pico-Sirius red, and imaged (×10). The red collagen stain was digitally quantified and plotted as fold increase relative to the sham levels adjusted to 1. Error bars represent SEM, *$P \leq 0.05$ vs. sham.

in PLCβ1 will potentially induces further down-regulation of miR-26b, creating a double-negative feedback loop that amplifies the PLCβ1 signalling pathway (*Figure 4H*).

3.5 Overexpression of miR-26b in the heart inhibits cardiac hypertrophy

Since miR-26a/b is down-regulated during cardiac hypertrophy, as its levels inversely correlate with its targets, we predicted that normalizing this decrease would inhibit up-regulation of GATA4 and, in turn, cardiac hypertrophy. To address this question, we developed a mouse overexpressing miR-26b in the heart. Two transgenic lines survived and developed normally. Endogenous miR-26b levels are relatively high in the adult tissue (as seen in *Figure 2*) and the lack of any significant effect of the transgene on baseline levels of GATA4 or PLCβ1 indicates that it may be saturating in the adult heart (*Figure 5F*), as similarly noted in the liver.[23] At 12 weeks, the transgenic mice (Tg), matched with same gender wild-type (wt) littermates, were subjected to TAC or a sham operation. We first confirmed that miR-26b was down-regulated in the wt heart after TAC (0.58 ± 0.25), while in the Tg mice, with 3.6 ± 1-fold higher miR-26b at baseline, it dropped to wt baseline levels (0.98 ± 0.2), thus normalizing its levels during TAC, as expected (*Figure 5A*). On the other hand, miR-21, which is consistently up-regulated during hypertrophy, was undisturbed by the transgene (*Figure 5B*), showing selectivity of miR-26b on hypertrophy-elicited changes. Heart weight/tibial length increased by $35 \pm 14\%$ after TAC in the wt mice, but was significantly dampened in the miR-26b-Tg (16 ± 7) (*Figure 5C*). Echocardiography confirmed TAC-induced thickening in the anterior and posterior ventricular walls (AW and PW, respectively) when measured during diastole (D) or systole (S), which was significantly reduced by the miR-26b-Tg. Note that this is an early stage of hypertrophy (2-week post-TAC), where there was no change in LV end-diastolic dimensions (LVEDD), LV end-systolic dimensions (LVESD) (*Figure 5D*), LV end-diastolic pressure, or ejection fraction (*Figure 5E*), in either wild-type or transgenic mice, reflecting normal function. In contrast to the heart weights, the mRNA of the hypertrophy markers, CARP, ANF, and αSkAc, increased equivalently in both the wt and Tg hearts (*Figure 5F*). The one exception, though, was βMyh, which was significantly lower in the miR-26b-Tg; this was further confirmed by western blot analysis with an anti-slow myosin antibody (*Figure 5G*). The western blot analysis also confirmed that the increase in GATA4 protein seen in the hypertrophied heart was almost completely abolished in the Tg mouse, while CARP and PLCβ1 were not. Thus, under these conditions, PLCβ1 expression must be regulated by other factors. Commensurate with the improvement in cardiac dysfunction and lower βMyh levels, the miR-26b transgenic heart also exhibited 40% less collagen deposition in the heart after TAC (*Figure 5H*). While the results prove the requirement of miR-26b down-regulation for the increases in GATA4, βMyh, and heart weight after TAC, suppression of miR-26b by an anti-miR was not sufficient for inducing cardiac hypertrophy (see Supplementary material online, *Figure S5*).

4. Discussion

GATA4 protein is higher in the neonatal and hypertrophied hearts, where it plays an essential role in development and hypertrophy.[10,24] Here, we show that while the GATA4 gene is more actively transcribed ($2\times$) during post-natal cardiac growth vs. the adult heart, its transcription and mRNA levels remained unchanged during pressure overload-induced cardiac hypertrophy in the mouse. In both cases, the protein level was higher, albeit to a greater extent, in the neonatal heart (approximately seven-fold) than in the TAC-induced hypertrophy (approximately three-fold) vs. the adult heart. The findings supported a post-transcriptional mode of regulation of GATA4 expression, in particular, after TAC. Further analysis revealed that this was a function of miR-26b, which is lower in the neonatal vs. adult tissues, and is down-regulated after TAC. Interestingly, the 3′UTR of GATA4 was reported to be a 'hotspot' for mutations associated with congenital heart diseases, most of which were predicted to result in misfolding of the first 500 bases of the 3′UTR,[25] which encompasses the miR-26b target site. This supports a post-transcriptional mode of regulation involving the 3′UTR.

Unlike TAC-induced cardiac hypertrophy, exercise-induced hypertrophy is associated with an increase in GATA4 mRNA as a result of down-regulation of c/EBPβ, which leads in enhanced serum response factor DNA-binding activity.[26] Similarly, we found that during normal post-natal hypertrophic growth, both *Gata4* transcription and mRNA levels were approximately two-fold higher than those measured in the adult heart. Furthermore, the neonatal hearts, but not the TAC-induced hearts, exhibited reduced transcription of the *Cebpb* compared with normal adult (see Supplementary material online, *Figure S6*). Notably, while GATA4 mRNA levels were approximately two-fold higher in the neonatal heart, protein levels were approximately seven-fold higher than adult levels, suggesting a superimposed post-transcriptional mechanism. Since miR-26b is also lower in the neonatal heart, we propose that it is also involved in the regulation of GATA4. This difference in the mechanisms regulating GATA4 in physiological vs. pathological models of hypertrophy might account for the more impressive increase in GATA4 protein seen in the former. In contrast, though, the *Carp* gene transcription, mRNA, and protein levels were equivalently up-regulated in either models of hypertrophy, reflecting the selective nature of the different modes of gene regulation.

miRNAs are characterized by their ability to simultaneously target multiple genes that are involved in the same function.[12] While we show here that miR-26b targets GATA4 and PLCβ1, we assume that there are other targets involved in mediating its effects. This could explain why our attempt to rescue the inhibitory effect of miR-26b on myocyte hypertrophy by overexpression of GATA4 was unsuccessful. Conversely, multiple miRNAs may simultaneously target a single gene, thus the translational output of that gene is the net result of their combined effects, which could explain why overexpression of miR-26b was not sufficient for inhibiting PLCβ1 expression during TAC. On the other hand, our results show that miR-26b is the main miRNA that impacts the translational levels of GATA4 both *in vitro* and *in vivo*. Indeed, acute knockdown of miR-26b in the heart resulted in ~2.5-fold increase in GATA4, which is the equivalent of what we observe during TAC. However, unlike a transgenic mouse model overexpressing GATA4 in the heart,[10] our miR-26b knockdown model did not exhibit any signs of cardiac hypertrophy within a 2-week period. The differences between the two models could be attributed to temporal differences in the increase in GATA4, the fact that the knockdown of miR-26b is systemic and that other cell types and organs may also exhibit an increase in GATA4 and indirectly impact hypertrophy, or that other miR-26b targets have a negative impact on hypertrophy. Further

studies are underway to determine whether the increase in GATA4 in this model is associated with an increase in its DNA binding to its target genes.

Finally, we conclude that during hypertrophy, some genes, like GATA4, are mainly regulated at the translational level via miRNA, with the advantage of circumventing a transcriptional step. One can then envision that a single miRNA has the capacity to induce synchronous and prompt regulation of a cadre of functionally related genes in response to a stimulus.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

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