

Promyelocytic Leukemia Zinc Finger Protein Activates GATA4 Transcription and Mediates Cardiac Hypertrophic Signaling from Angiotensin II Receptor 2

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Abstract

Background: Pressure overload and prolonged angiotensin II (Ang II) infusion elicit cardiac hypertrophy in Ang II receptor 1 (AT₁) null mouse, whereas Ang II receptor 2 (AT₂) gene deletion abolishes the hypertrophic response. The roles and signals of the cardiac AT₂ receptor still remain unsettled. Promyelocytic leukemia zinc finger protein (PLZF) was shown to bind to the AT₂ receptor and transmit the hypertrophic signal. Using PLZF knockout mice we directed our studies on the function of PLZF concerning the cardiac specific transcription factor GATA4, and GATA4 targets.

Methodology and Principal Findings: PLZF knockout and age-matched wild-type (WT) mice were treated with Ang II, infused at a rate of 4.2 ng·kg⁻¹·min⁻¹ for 3 weeks. Ang II elevated systolic blood pressure to comparable levels in PLZF knockout and WT mice (140 mmHg). WT mice developed prominent cardiac hypertrophy and fibrosis after Ang II infusion. In contrast, there was no obvious cardiac hypertrophy or fibrosis in PLZF knockout mice. An AT₂ receptor blocker given to Ang II-infused wild type mice prevented hypertrophy, verifying the role of AT₂ receptor for cardiac hypertrophy. Chromatin immunoprecipitation and electrophoretic mobility shift assay showed that PLZF bound to the GATA4 gene regulatory region. A Luciferase assay verified that PLZF up-regulated GATA4 gene expression and the absence of PLZF expression in vivo produced a corresponding repression of GATA4 protein.

Conclusions: PLZF is an important AT₂ receptor binding protein in mediating Ang II induced cardiac hypertrophy through an AT₂ receptor-dependent signal pathway. The angiotensin II-AT₂-PLZF-GATA4 signal may further augment Ang II induced pathological effects on cardiomyocytes.

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Introduction

Angiotensin (Ang) II is a potent vasoactive peptide, with strong effects on cardiac hypertrophy and congestive heart failure. Ang II has direct effects on elevated blood pressure, transactivation of the EGF receptor, and generation of reactive oxygen species [1]. Ang II binds to two major receptor subtypes, AT₁ and AT₂, with the most noted physiological and pathophysiological actions through the AT₁ receptor. However, the AT₂ receptor signaling and its significance have become more important, especially regarding cardiac remodeling mechanisms which are under-defined [2]. AT₂ receptor expression is generally high in fetal tissues, declines rapidly postnatal to low levels in specific tissues, and then is re-expressed in certain pathological conditions such as cardiac hypertrophy, strongly suggesting important roles of the AT₂ receptor in tissue growth and remodeling [3].

Mechanical stress alone induces cardiac hypertrophy in vivo [4]. Pressure overload elicits ventricular hypertrophy in AT_{1a} null mice

[5,6,7,8]. By contrast, in AT₂ null mice with intact AT₁ pressure overload or chronic Ang II infusion fails to elicit cardiac hypertrophy and interstitial fibrosis [9,10]. Transplantation of wild type kidney to AT_{1a} null mice and subsequent Ang II infusion result in hypertension and cardiac hypertrophy indicating exclusive roles of the kidney in the etiology of hypertension [11] and a potential role of AT₂ in cardiac hypertrophy. Also, the transfection of the AT₂ receptor into cultured neonatal cardiomyocytes induces hypertrophy [12]. These results imply that in the heart Ang II activates AT₂ to transmit a hypertrophic signal. This contrasts with other tissues where AT₂ has been shown to elicit antigrowth and pro-apoptotic signals.

A widely accepted AT₂ antihypertrophic signaling mechanism is a direct G-protein independent activation by AT₂ of the protein tyrosine phosphatase SHP-1 that blocks growth factor signals [13,14].

In search of the molecular mechanism which may provide material support for the unique cardiac hypertrophic response to

the AT₂ receptor action *in vivo*, we examined the hypothesis that a specific AT₂-binding or modulating protein exists in the heart. The transcription factor promyelocytic leukemia zinc finger protein (PLZF) acts as a binding partner to the C-terminus of the AT₂ receptor which was revealed by the yeast two-hybrid system and affinity binding technique. PLZF highly expressed in the heart activated gene transcription of the P85 α regulatory subunit of phosphatidylinositol 3 kinase (p85 α -PI3K). P85 α further activated its downstream kinases Akt/PKB and p70^{S6k} resulting in cardiac hypertrophy [15].

PLZF is a transcription factor which contains 9 zinc fingers in the carboxyl terminal area and its amino terminus BTB/POZ domain mediates most biological functions of the zinc finger protein [16]. As an important transcription factor in cell differentiation and development, PLZF exhibits proapoptotic function in limb development [17] and an antiapoptotic role in developing testis [18]. PLZF regulates cyclin A2, c-myc, kit gene expression [19,20,21] and is involved in the signal of AT₂ [15] and renin receptors [22].

The function of PLZF in different tissues and cells depends on its specific gene sequence context and different functional interaction partners [23,24,25].

Several cardiac transcription factors involved in fetal heart development have been identified including GATA4,5,6, NFAT 3, Nkx 2.5 and the transcription factor regulator HDACs [26,27,28,29,30,31]. A reversion of these fetal gene expressions leads to maladaptive heart function [32]. Given that PLZF is an AT₂ receptor binding protein in the heart; we hypothesized that it can interact with some of these transcription factors, specifically GATA4 in the heart. In the present study, we employed PLZF^{-/-} mice to consolidate the hypothesis that the cardiac hypertrophic action of AT₂ is regulated by PLZF *in vivo*.

Methods

Animals

All of the animal experimental protocols were approved by the Vanderbilt University Animal Use and Care Committee (A3227-01). *Agtr 2-/-* mice were generated as described [33] and backcrossed to C57BL/6 genetic background. PLZF deficient mice were prepared by targeted disruption of the gene *Zfp145* in embryonic stem cells as described [18], they were backcrossed to C57BL/6 background. Because PLZF^{-/-} animals are defective in hind limb bone formation, their access to chow was facilitated by providing food in a dish placed at the floor level. Ten to twelve weeks old PLZF^{-/-} (n = 12) and WT (n = 10) male mice were used; their body weight is shown in Figure 1. In a pentobarbital-anesthetized (10 mg/kg I.P) mouse, an Ang II-impregnated pellet (Innovative Research of America) was placed under the shoulder skin. The pellets were prepared to release Ang II at a rate of 4.2 ng kg⁻¹ min⁻¹ for 21 or 60 days. For control, saline pellets were implanted. For the hydralazine group, wild type mice receiving Ang II pellets were given hydralazine 500 μ g/ml in drinking water. For the AT₂ receptor blockade group, wild type mice received a pellet which releases the AT₂ blocker PD123319 at a rate of 15 μ g kg⁻¹ min⁻¹ together with the Ang II pellet, as above.

Blood Pressure Measurement

Mouse systolic blood pressure (SBP) was measured by the tail-cuff method and random samples (n = 6–7/group) verified the SBP by carotid arterial catheterization method. We trained mice for the manometry daily for 1 week prior to the experiment. Blood pressure was measured at 0, 7, 14, and 21 days during Ang II infusion.

Echocardiography

Transthoracic echocardiography was performed using VisualSonics Vevo 770 High-Resolution Imaging System (VisualSonics Inc.) or Sonos 5500 (Agilent) for measurements of left ventricle (LV) internal diameter at end diastole and end systole, interventricular septal wall thickness (IVS) and posterior wall thickness (PW) as previously described [5].

Histochemistry

Mouse hearts were isolated after perfusion with 40 mM KCl, and fixed in 4% paraformaldehyde with PBS. Four μ m paraffin sections were cut and stained with hematoxylin-eosin solution. The collagen fraction was calculated as ratio of the sum of the total area of interstitial fibrosis to the sum of the total connective tissue area plus the myocyte area in the entire visual field of a section. The area of perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area. Approximately 100 cells were examined in each heart.

Western Blot

Mouse ventricles were ground on dry ice, or in liquid nitrogen in a pestle and mortar into a fine powder and homogenized in 1 mL of ice-cold TNF buffer (20 mM Tris-HCl (pH: 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 50 mM NaF, 25 μ g/ml aprotinin, 25 μ g/ml leupeptin, 1 mM Na₃VO₄, 1 mM PMSF and 5 mM 2-mercaptoethanol). After 30 minutes exposure at 4°C, homogenates were centrifuged at 30,000 \times g for 30 minutes and supernatants were saved. Cardiac extracts were subjected to SDS-PAGE and transblotted onto PVDF membranes. Membranes were blocked, washed and incubated with primary and secondary antibodies. Protein bands were visualized by enhanced chemiluminescence (ECL) plus detection system.

Competitive Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Mouse ventricles were extracted in RNeasy Fibrous Tissue Mini kit (Qiagen). mRNA for AT1 and AT2 were determined by RT-PCR, according to the published method [34].

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts (10 μ g) from COS7 cells that were transfected with pCDNA4-PLZF plasmid was incubated with 2 nM ³²P-labeled double-stranded oligonucleotide for 20 min at room temperature. Mouse anti-PLZF antibody (Santa Cruz Biotechnology, Santa Cruz, California) (2 μ g) was added for the supershift assay. Protein-DNA complexes were electrophoresed in 4% native polyacrylamide gels and autoradiographed. The sense strands of the oligonucleotides used in the EMSA were: 5'GGACAATC-TAAAGTTCTTTCT-3' (GATA4 native), and 5'GGACAAT-CATATGTTCTTTCT-3' (GATA4 mutated), based on the GATA4 gene sequence 3 (NC_000008 NCBI).

Chromatin Immunoprecipitation (ChIPs) Assay

ChIPs analysis was carried out with a commercial kit (Upstate Biotechnology) with some modifications to the manufacturer's recommended conditions. Briefly, 2 \times 10⁶ CHO-K1 cells, stably expressing AT₂ and PLZF were cultured for 2 days and treated with 1% formaldehyde for protein-DNA cross-linking. The nuclear pellet was suspended in 600 μ l of lysis buffer containing 1% SDS and 1 \times protease inhibitor cocktail, incubated for 10 min on ice, and sonicated sufficiently to shear the DNA to an average size of 500 to 1,000 bp. Chromatin was diluted 10-fold with ChIP dilution buffer, precleared with 80 μ l of protein A-agarose and

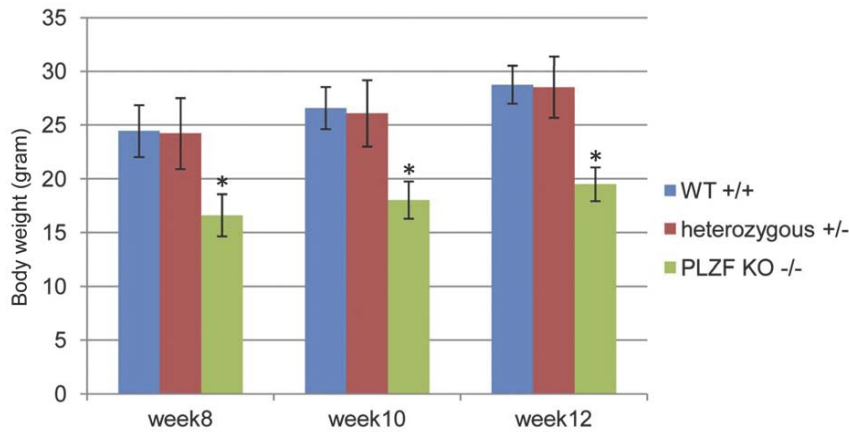


Figure 1. PLZF^{-/-} mice weigh less than their wild type and heterozygote siblings. Mice were weighed for comparison. (* indicates $p < 0.05$ vs all other groups; $n = 12$; data are mean \pm S.E.M). doi:10.1371/journal.pone.0035632.g001

incubated with the anti-PLZF antibodies on a rotating shaker at 4°C overnight. After protein removal purified DNA was resuspended in 20 μ l of H₂O and analyzed by PCR. DNA for the input control was diluted 1:100 before the PCR. Reactions were carried out in a volume of 50 μ l, with initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 1 min, and extension at 72°C for 40 sec, followed by a 7-min terminal extension at 72°C.

Luciferase Assay

Human *GATA4* gene 5' upstream regulatory sequence was amplified by PCR with primers 5'-GGCATTGTA-CATTCTTCTCA-3'/5'-ACCTATTGGGGGCAGAAGC-3', and was cut by KpnI and Bgl II. The promoter region of the *GATA4* gene was amplified with primers 5'GTAGCG-CACGTCTCTTTCC-3'/5'GGTAGCACTTGGGCATTTTC-3', and was cut by Bgl II and Hind III. These fragments were inserted to PGL3 basic vector plasmid (Promega). The dual luciferase assay system (Promega) was used to normalize for transfection efficiency by Renilla luciferase activity.

Statistics

Data are expressed as mean \pm SEM. The significance of differences between control and experimental groups were evaluated using a one-way ANOVA with Student Newman-Keuls test. $P < 0.05$ was considered statistically significant.

Results

Reduced Cardiac Hypertrophy and Fibrosis in Ang II-treated Homozygous PLZF^{-/-} Mice

We reported that prolonged Ang II infusion [9] or pressure overload [10] failed to cause cardiac hypertrophy in AT₂ deficient mice. We also reported that in cells and tissues high in PLZF expression the AT₂ signal was transduced by PLZF [15]. We treated PLZF^{-/-} and WT male mice with Ang II or with saline for 3 weeks by continuous infusion from subcutaneously embedded Ang II impregnated pellets to investigate the role of PLZF in Ang II induced cardiac hypertrophy. The systolic blood pressures of WT and PLZF^{-/-} mice with Ang II infusion was raised to 140 mmHg (Figure 2A). The wild type mice showed a robust increase in the heart weight/body weight ratio whereas PLZF^{-/-} mice showed little change in the ratio. (Figure 2 B,C).

The Ang II treatment significantly increased wall thickness of IVS and PW in WT mice, but no obvious change was detected in PLZF^{-/-} mice (Figure 2, D, E). Masson's staining revealed that both interstitial and perivascular fibrosis (Figure 3,A,B) were significantly increased by Ang II infusion in WT mice, and no significant increase was seen in PLZF^{-/-} mice after the Ang II infusion. Taken together, the reduced cardiac hypertrophy and fibrosis indicate that PLZF is one of the transcription factors mediating Ang II-induced cardiovascular remodeling.

AT₂ Receptor Pathway is Necessary for Ang II Induced Cardiac Hypertrophy

We performed a series of *in vivo* studies, including wild type mice with control pellet, wild type mice with Ang II infusion, wild type mice with Ang II plus hydralazine, wild type mice with Ang II plus PD123319 and AT₂ knockout mice with Ang II (Figure 4, A,B,C,D) to distinguish the functional roles of AT₂ and pressure load in cardiac hypertrophy. We found that co-infusion of the specific AT₂ blocker PD123319 with Ang II in wild type C57BL/6 mice suppressed Ang II-induced cardiac hypertrophy (Figure 4 B,C,D) confirming that AT₂ is responsible for Ang II-stimulated hypertrophy. Ang II infusion into both wild type and AT₂ knockout mice led to sustained elevation in systolic blood pressure (~140 mmHg) which was normalized to 103–106 mmHg by a non-specific vasodilator hydralazine (Figure 4A). The hydralazine administration abolished Ang II induced cardiac hypertrophy (Figure 4 B,C,D). These results suggest that the elevated blood pressure is an important component of the increased hypertrophic response in AT₂-dependent Ang II signals.

AT₂ Expression in Cardiac Ventricles is Promoted by Ang II Infusion in Wild Type Mice

Given evidence for the role of AT₂ in ventricular hypertrophy in wild type mice receiving Ang II infusion (Fig. 4 B,C,D) we examined possible changes in expression levels of AT₁ and AT₂ during infusion of Ang II. *In vivo* cardiac AT₂ receptor protein is expressed at about 20% of AT₁ protein level in untreated C57BL6 mouse strain which is too low to be determined by competitive ligand binding assay [34]. By RT-PCR AT₂ mRNA expression increased by 80% in 3 days and 180% in 7 days with \pm 15% error, $n = 4$. Whereas AT₁ mRNA did not increase in the first 3 days and showed a 100% increase in 7 days (with \pm 19% error, $n = 4$). Almost 3-fold increase in AT₂ mRNA suggests that Ang II will

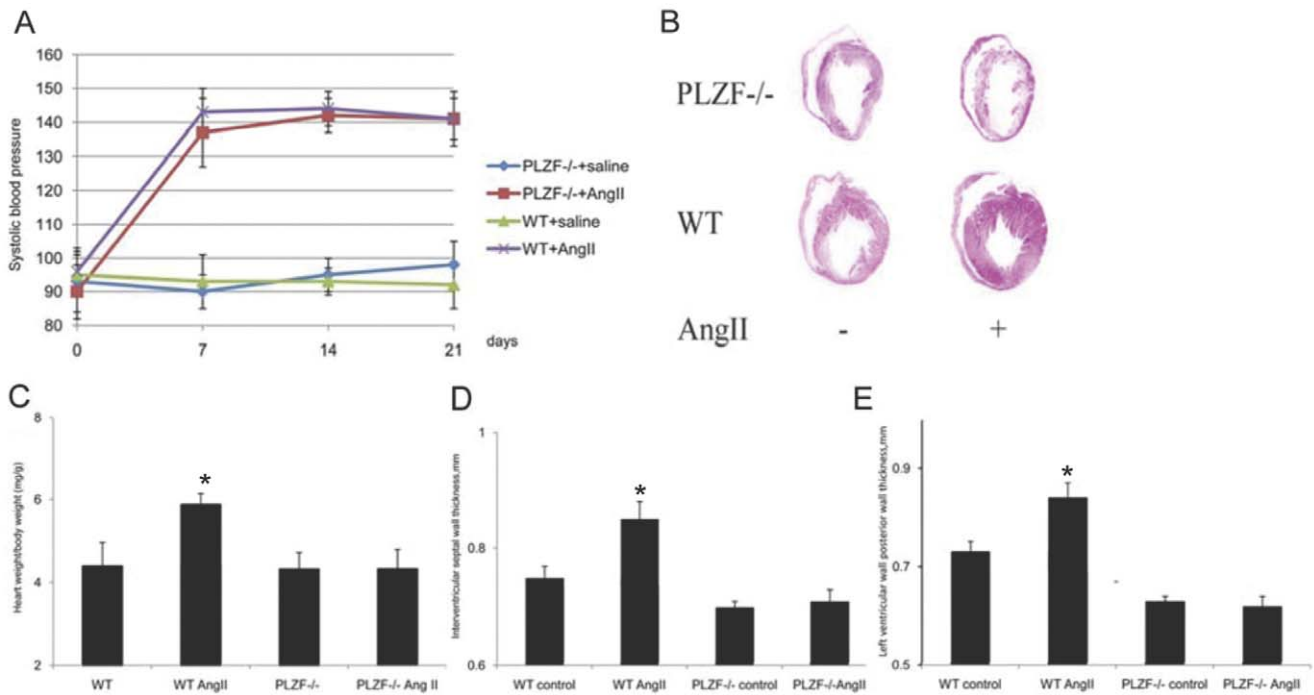


Figure 2. PLZF^{-/-} mice show no obvious cardiac hypertrophy by Ang II infusion. (A) Systolic blood pressure increased in Ang II group (* indicates $p < 0.05$ vs vehicle groups; $n = 12$; data are mean \pm S.E.M.). (B) Mouse heart cross-section of the left ventricle. (C) The bar graph shows the ratio of heart weight (mg) to body weight (g) of mice from each group (* indicates $p < 0.05$ vs all other groups; $n = 8$; data are mean \pm S.E.M.). (D)(E) Echocardiography data, (D) interventricular septal (IVS) and (E) left ventricular posterior wall (LVPW) thickness (* indicates $p < 0.05$ vs all other groups; $n = 8$; data are mean \pm S.E.M.). doi:10.1371/journal.pone.0035632.g002

have a fairly strong effect on downstream events of the heart. These results are basically in agreement with observations reported by Fujii on double transgenic mouse expressing human renin and angiotensinogen which develops angiotensin dependent hypertension [34].

PLZF Specifically Binds to the Upstream Flanking Region of *GATA4* Gene

We previously reported that the AT_2 -PLZF-p85 α -PI3K-Akt mechanism was involved in the AT_2 mediated signaling pathway [15]. Considering that PLZF is an important transcription factor in development [17,18] and cardiac hypertrophy [15,22], we further studied the possibility of a transcription function of PLZF. According to Li et al. [36], a core consensus sequence for the specific binding of the PLZF is AT/GG/CTA/CA/CAGT. Based on MatInspector reports [37], a putative PLZF binding site is present in the human *GATA4* promoter region with a high core and matrix similarity (Sequence from NC_000008 NCBI). We performed an electrophoretic mobility shift assay (EMSA) to investigate whether it is a PLZF target. The results showed a positive band that was abolished by a competing mutant probe. Supershift assay with PLZF antibody showed a retarded band, which further confirmed the binding activity (Figure 5A). These results indicate the potential for PLZF to bind to the *GATA4* gene regulatory region. Further, chromatin immunoprecipitation assay (ChIPs) analysis verified that this *GATA4* gene sequence at the chromatin level was indeed occupied by PLZF. A CHO-K1- AT_2 -PLZF cell line with stable AT_2 and PLZF expression was cultured for 2 days and treated with 1% formaldehyde to cross-link DNA and associated proteins. The chromatin was fragmented by sonication. The resulting PLZF-bound fragments

were subjected to immunoprecipitation with PLZF antibody, and the chromatin-bound proteins were digested overnight with proteinase K in 200 mM NaCl at 65°C. DNA freed from the bound protein was identified by PCR which was performed with primers encompassing the putative PLZF binding sequence. As shown in Figure 5B, this *GATA4* regulatory sequence was bound by PLZF.

Upregulation of *GATA4* Gene Expression through the AT_2 and PLZF Signaling Pathway

The present finding that the *GATA4* gene has a specific PLZF binding site in its upstream flanking region suggests that it may be a functional site that regulates transcription of the *GATA4* gene. Since we have found that Ang II-mediated cardiac hypertrophy depends on the expression of PLZF, and that PLZF activation is driven by AT_2 in the heart [15]; and since *GATA4* is one of the well known factors involved in cardiac development and hypertrophy [26], we examined the possible transcriptional role of PLZF on *GATA4* gene expression by luciferase assay in R3T3 cells which express the AT_2 receptor but not AT_1 . The luciferase plasmid construction was based on pGL3-basal vector with a 904 bp insert from the upstream regulatory sequence of the human *GATA4* gene and a *GATA4* promoter [35]. As shown in Figure 6A, PLZF-expressing R3T3 cells had a markedly elevated (200% of the basal level) luciferase transcription activity. When Ang II (0.1 mM) was added to the PLZF-transfected R3T3 cells, the luciferase activity was further elevated to 300% of the control level. These results support the hypothesis that PLZF up-regulates *GATA4* gene expression, and that the activation is further enhanced by Ang II action through the AT_2 receptor.

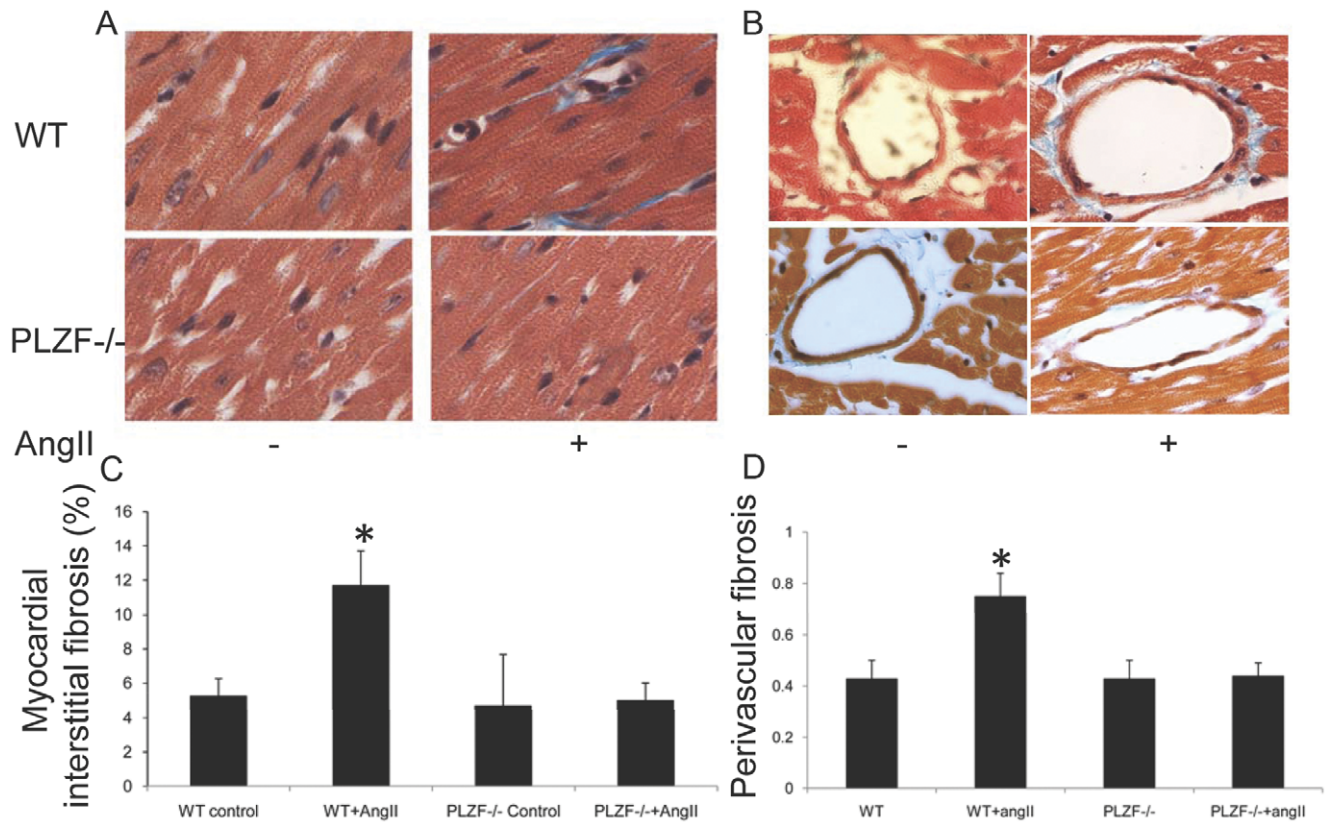


Figure 3. Less fibrosis is observed in Ang II infused PLZF^{-/-} than wild type mice. Sections of left ventricle were stained with Masson's trichrome to determine fibrosis. (A) Interstitial fibrosis. (* indicates significant changes at $p < 0.05$ vs all other groups; $n = 100$; 5 mice for each group; data are mean \pm S.E.M.). (B) Perivascular fibrosis. (* indicates $p < 0.05$ vs all other groups; $n = 100$; 5 mice for each group; data are mean \pm S.E.M.). doi:10.1371/journal.pone.0035632.g003

Results in Figure 6B further confirm this conclusion as the non-peptide AT₂ receptor antagonist PD123319 completely eliminated the enhanced effect of Ang II on luciferase activity.

Elevated Expression of GATA4 Protein in Ang II-treated Wild Type Mouse Heart

Given the ability of PLZF to increase GATA4 gene expression *in vitro*, we next examined whether the absence of PLZF expression *in vivo* produced a corresponding repression of GATA4 protein. PLZF^{-/-} and wild type mice received Ang II infusion for 3 weeks. GATA4 protein was examined in the left ventricular extract by Western blotting using GATA4 polyclonal antibodies. GATA4 protein was markedly elevated after 3 weeks in WT mice, whereas in PLZF^{-/-} no significant induction of GATA4 protein was recognized, as shown in Figure 7. This result provides further support to the conclusion that the *GATA4* gene is regulated by PLZF *in vivo*.

Ang II Activates Cardiac GATA4 Signal to Target Genes

GATA4 controls numerous cardiac target genes. The question arose whether the upstream signal of the Ang II-AT₂-PLZF signal generally reaches GATA4. To test such a possibility, we studied whether Ang II infusion for 3 weeks to wild type C57BL/6 mouse will stimulate synthesis of well known targets of GATA4 pro-atrial natriuretic factor (pro-ANF) [38] and RhoA [39]. Ang II infusion markedly increased ventricular pro ANF compared with controls ($n = 3$) by Western blot analysis of the target proteins (Figure 8).

These results show Ang II signal, possibly via AT₂, activates GATA4 target genes.

Discussion

In the present study, we report evidence indicating PLZF, a Kruppel-like zinc finger protein highly expressed in the heart [40], is a crucial transcription factor that regulates cardiac hypertrophy through the AT₂ receptor in response to Ang II. Ang II-activated AT₂ receptor has been shown to bind PLZF and facilitate its nuclear translocation [15]. The present study shows that it up-regulates the expression of GATA4, a key cardiac morphogenic, hypertrophy and remodeling regulator [41].

PLZF was first recognized to fuse with retinoic acid receptor RAR α in acute promyelocytic leukemia. PLZF suppresses gene transcription by recruiting corepressors to the gene regulation region and activate gene transcription with different molecular mechanism which has not been well defined [25]. PLZF is increasingly recognized as a key regulator in cell differentiation, growth or self-renewal process. Using PLZF knockout mice, we found suppressed cardiac hypertrophy (Fig. 2 B,C,D,E) and fibrosis (Fig. 3 A,B,C,D) in PLZF^{-/-} mice subjected to chronic Ang II stimulation. Further experiments revealed PLZF regulated GATA4 expression, a vital factor in heart development and differentiation and remodeling [26,41,42]. GATA4 directly stimulates numerous cardiac-specific genes, including those of α - and β -myosin heavy chain genes which are key indices of cardiomyocyte hypertrophy [43,44,45]. Therefore, we believe that the expression of GATA4 directly regulated by PLZF is

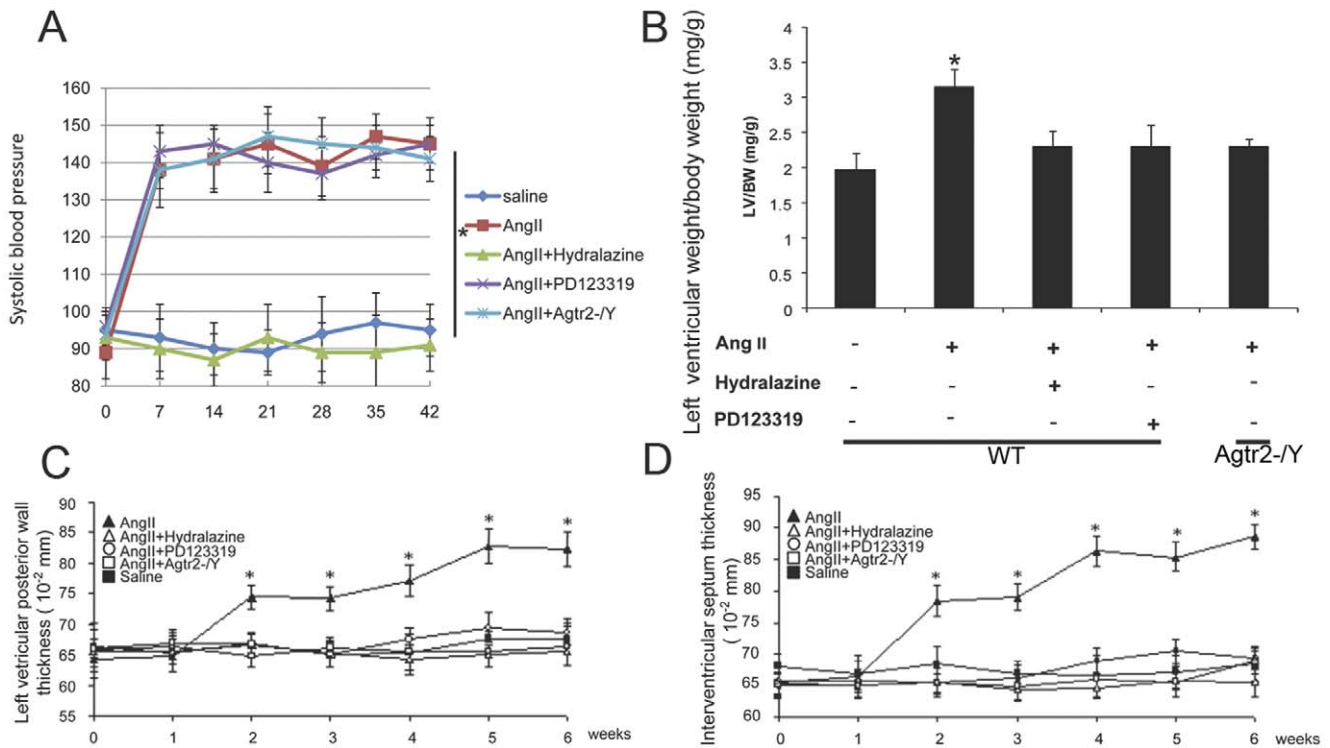


Figure 4. Elevated blood pressure and intact AT₂ receptor are involved in eliciting Ang II-induced cardiac hypertrophy. (A) Systolic blood pressure increased in Ang II group, Ang II + PD123319 group and Ang II+ *Agtr2*-Y group (*indicates p<0.05 versus vehicle; n=15; data are mean±S.E.M.). (B) The bar graph shows the ratio of left ventricular weight (mg) to body weight (g) of mice from each group (* indicates p<0.05 vs all other groups; n=7; data are mean±S.E.M.). (C)(D) Echocardiography data, line graphs show changes in IVS (C) and LVPW (D) in mice of each group (* indicates p<0.05 vs all other groups; n=15; data are mean±S.E.M.). doi:10.1371/journal.pone.0035632.g004

another important factor of the cardiac remodeling controlled by the transcription factor PLZF in the Ang II AT₂ signaling pathway.

Senbonmatsu, et al previously showed that stimulation of the AT₂ receptor [15] in cells expressing PLZF, such as cardiomyocytes, elicits binding of PLZF to the receptor and its subsequent translocation into the nucleus, where it up-regulates the p85 α regulatory subunit of PI3K. The nuclear translocation was demonstrated by markedly increased PLZF in cardiocyte nuclei

(by immunohistochemical staining) of Ang II-infused wild type mice and also in AT₂-expressing R3T3 cells transfected with a PLZF-expression plasmid [15]. It is this AT₂-mediated nuclear translocation of PLZF that accounts for the activation of p85 α in the previous studies [15] and GATA4 in the present study. The activation of the PI3K/Akt signal leads to cellular hypertrophy due to stimulation of protein synthesis by p70^{S6k}. Moreover, GSK3 β is another important downstream hypertrophic factor in the PI3K/Akt signal pathway. GSK3 β phosphorylates GATA4 to

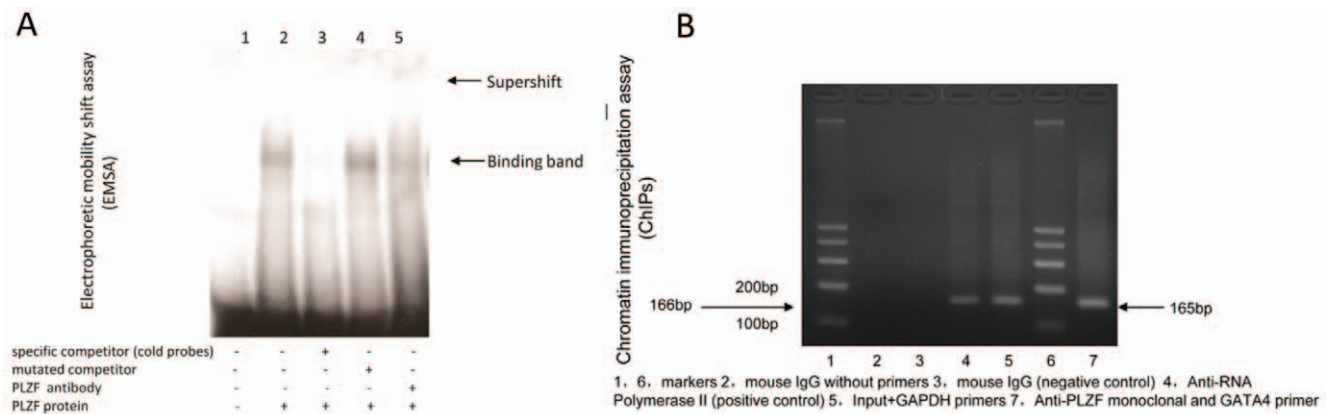


Figure 5. PLZF binds to the regulatory region of GATA4 gene. (A) Electrophoretic mobility shift assay (EMSA) verified the PLZF binding activity at GATA4 gene. Lane 1: negative control with nuclear extract of COS7 cells not expressing PLZF. Lane 2: positive control with PLZF expressing cell nuclear extract. (B)Chromatin immunoprecipitation assay (ChIPs) analysis determined the PLZF occupancy in GATA4 gene at the chromatin level. doi:10.1371/journal.pone.0035632.g005

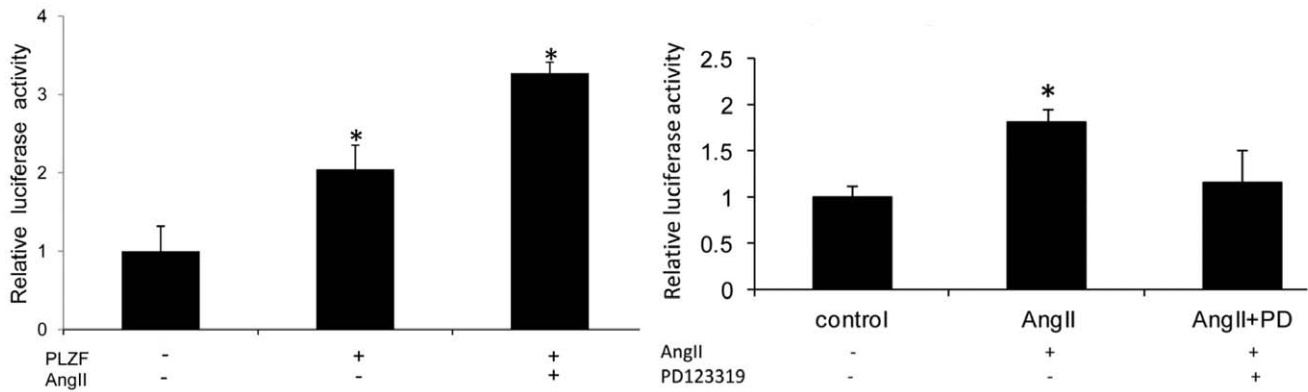


Figure 6. PLZF up-regulates GATA4 gene expression. (A) Luciferase reporter gene activity in PLZF co-transfected R3T3 cells (* indicates $p < 0.05$ vs control group; $n = 5$; data are mean \pm S.E.M.). (B) Luciferase reporter gene activity in R3T3 cells which were pretreated with AT_2 antagonist PD123319 for 1 hour before experiment. (* indicates $p < 0.05$ vs all other groups; $n = 5$; data are mean \pm S.E.M.). doi:10.1371/journal.pone.0035632.g006

promote its export from the nucleus through the exportin, Crml [46]. Akt inhibits GSK3B activity and enhances nuclear accumulation of GATA4. Taken together the Ang II- AT_2 exerted multiple convergent effects directed to cardiac hypertrophy through activation of $p85\alpha$ PI3K and $p70^{S6k}$, inhibition of GSK3B to facilitate nuclear localization of GATA4 and stimulation of GATA4 transcription.

The role of the AT_2 receptor in cardiac hypertrophy is not completely explained. The present findings that two of the arbitrarily selected GATA4 targets are activated by Ang II,

presumably via AT_2 -PLZF system, seems to indicate that many of GATA4 targets may be activated by this mechanism. More detailed studies are necessary to generalize it as a new GATA activation mechanism. The AT_2 initiated GATA4 activation and cardiac hypertrophy are novel and somewhat surprising in view of the generally recognized AT_2 function directed to growth inhibition. However, since the present hypertrophic mechanism involves cardiac specific GATA4 and heart selectively (but not exclusively) expressed PLZF, it may be a uniquely heart specific response under *in vivo* condition. Both AT_1 and AT_2 are

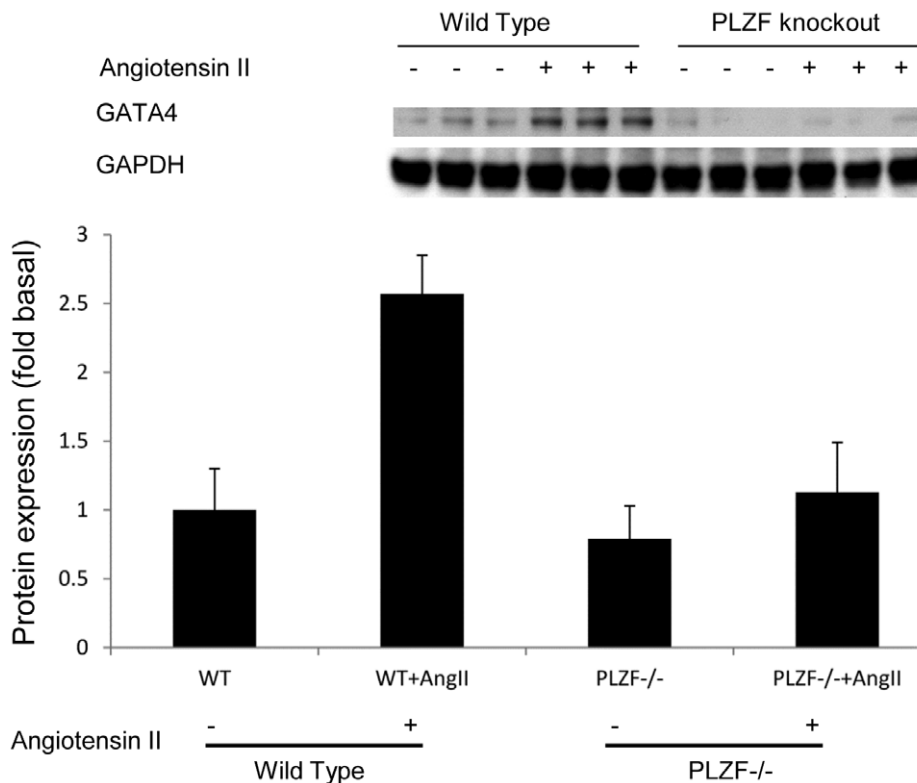


Figure 7. GATA4 protein expression is significantly elevated in the Ang II treated wild type mouse heart. Protein extracts from mice left ventricles were measured by Western blot (* indicates $p < 0.05$ vs all other groups; $n = 5$; data are mean \pm S.E.M.). Ordinate axis indicates protein amount normalized by GAPDH, control is set to 1.0. doi:10.1371/journal.pone.0035632.g007

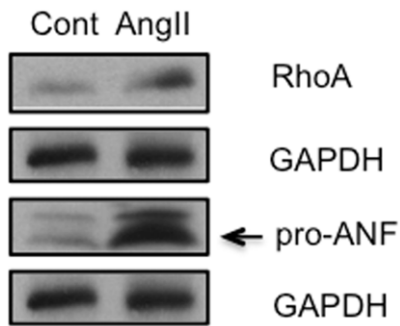


Figure 8. Ang II treatment of wild type mice for 3 weeks up regulates cardiac contents of RhoA and proANF, which are randomly selected targets of GATA4.

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proportionally up-regulated in the hypertrophic heart [34,47]. AT_1 inhibition does not prevent left ventricular remodeling induced by pacing [48]. Pressure overload elicits ventricular hypertrophy in AT_{1a} null mice [5,6,7,8]. By contrast, in AT_2 null mice pressure overload or chronic Ang II infusion fails to elicit cardiac hypertrophy and interstitial fibrosis [9,10]. Overexpression of AT_2 receptor in transgenic mice induces left ventricular hypertrophy [49]. Ang II up-regulates the immediate early transcription factor ATF3 through AT_2 signal pathway and induces left atrial hypertrophy [50]. Alternatively, deletion of either AT_1 or AT_2 markedly attenuates cardiac hypertrophy in natriuretic peptide receptor/guanylyl cyclase-A (GCA)-deficient mice [51]. Similar to these observations, our *in vivo* studies showed high blood pressure and AT_2 were critical for Ang II induced cardiac hypertrophy. Abating pressure overload by hydralazine, blockade of AT_2 receptor with the AT_2 antagonist PD123319 or deletion of AT_2 (*Agr2-1*) prevents cardiac hypertrophy.

Cardiac AT_2 is up-regulated by mechanical stretch [52] and pressure overload [34,53]. Moreover, AT_2 promotes ligand-independent, constitutive cardiomyocyte hypertrophy [54]. AT_1 and AT_2 receptors are considered to interact with each other to enhance the effects they mediate. Combined treatment with losartan and PD123319 proved to be more effective in attenuating the reflex increase in plasma adrenaline concentrations during insulin-induced hypoglycemia than either of the two Ang II

receptor antagonists given alone [55]. Furthermore, the combination of both AT_1 and AT_2 receptor antagonists, at concentrations that each partly reduced inositol 1,4,5-trisphosphate (IP_3), completely inhibited IP_3 formation, suggesting that AT_1 and AT_2 cooperate in Ang II-mediated IP_3 signal transduction for the actions of Ang II mediated by the IP_3 signal transduction pathway [56].

Because GATA4 with AP-1 (activator of protein-1) up-regulates AT_1 receptor expression [57], our study provides insight into a plausible mechanism of interaction between AT_1 and AT_2 signaling pathways, in which PLZF bridges AT_2 and AT_1 signaling through GATA4. Thus, AT_2 could be an upstream cardiac hypertrophy factor of AT_1 signaling. The interactions between AT_1 and AT_2 are complicated but the comprehensive understanding of these mechanisms could lead to better understanding of the therapeutic strategies of hypertension and cardiac hypertrophy. The present observation that AT_2 receptor antagonist PD123319 inhibits the Ang II mediated cardiac hypertrophy is in agreement with our earlier observation with AT_2 deficient mice which lost hypertrophic response to Ang II. It is important to note that normalization of blood pressure by hydralazine prevented cardiac hypertrophy (Fig. 2A) which indicates that pressure load is essential for the hypertrophy in agreement with existing reports [4,34,47,57] as discussed above myocyte stretch and tension induce expression of AT_2 [52,53].

In summary, the present results of *in vivo* and *in vitro* studies demonstrate that PLZF activates GATA4 gene transcription and plays a significant role in the AT_2 -mediated cardiac hypertrophic response to Ang II.

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Author Contributions

Conceived and designed the experiments: TI NW TS EL. Performed the experiments: NW RD AR. Analyzed the data: NW RD GF EL. Contributed reagents/materials/analysis tools: AR RD NW. Wrote the paper: TI NW GF EL. Critical revision: TI EL GF. Did mouse physiology of cardiac hypertrophy: ZT. Provided PLZF knockout mice and advised us on their breeding and selection: PPP.

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