ることから心拍出量の測定はFick法を使用した方がよい．右房圧や肺血管抵抗などが予後と密接な関係があることが知られており[7]，確定診断のためにも最も重要な検査手段となる．

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シンポジウム エポプロステノール治療—10年の歩み— 4

膠原病性肺高血圧症に対するエポプロステノールの使用経験

片岡 雅晴[1)] 佐藤 徹[1)] 吉野 秀朗[1)] 川上 崇史[2)]
田村 雄一[2)]

はじめに

膠原病性肺高血圧症の病態は，①免疫異常による血管炎が誘因と考えられる特発性肺動脈性肺高血圧症(IPAH)様のもの，②抗リン脂質抗体症候群などによる慢性肺血栓塞栓症に続発するもの，③エンドセリン-1産生亢進，VEGF受容体異常，遺伝子多型によるNOS産生低下などの生理学的異常によるもの，④膠原病に続発する間質性肺炎によるものなどが文献的に報告されている．

従来の治療
1．生活様式 運動量の制限，女性の妊娠の禁止
2．右心不全
ジゴキシン
利尿薬(フロセミド，スピロノラクトン)
3．低酸素血症－酸素吸入
4．抗凝固薬－ワルファリン
5．血管拡張薬
新しい治療
1）Ca blocker(5％に有効)
2）long acting beraprost
3）epoprostenol(PGI_2)持続注入
4）bosentan(エンドセリン拮抗薬)内服
5）sildenafil(PDE阻害薬)内服
6．移植－5年生存率50％
生体・両肺・心肺移植・片肺
これからの治療 7．血管増殖抑制薬 imatinib

図1 肺動脈性肺高血圧症の治療

膠原病の活動性と治療法

膠原病性肺高血圧症に対する治療法は，膠原病の疾患活動性の有無で分類し，活動性があれば膠原病の原病治療を優先し，それによって肺高血圧症が改善する場合もあるとされている．

1．膠原病の活動性を有する自験例

自験例を示す．症例は23歳，女性．主訴は労作時息切れである．半年前より微熱，関節痛を来し，1カ月前には息切れ，咳が出現し，徐々に増悪した．1週間前に近医で心拡大，心エコーで肺高血圧症を指摘された．家族歴，既往歴に特記すべきことなく，全身性エリテマトーデス(SLE)に続発する肺高血圧症と診断した．右心カテーテル検査で肺血管抵抗(PVR)は4.3 wood unit，平均肺動脈(PA)圧50 mmHgの症例であった．

ドブタミン投与開始後，ステロイドパルス療法，免疫抑制薬などを投与したところ，肺高血圧症が著明に改善した．

入院時心電図では右軸偏位が強く，右室負荷が認められたが治療によって改善がみられた．

2．活動性のない膠原病性肺高血圧症治療

活動性のない膠原病性肺高血圧症治療では，まず免疫抑制療法を行う．Jaisら[1)]はSLE，ないし混合性結合組織病(MCTD)に続発する肺動脈性肺高血圧症(PAH)症例23例に免疫抑制療法を行った．免疫抑制薬単独投与16例中8例と半数に効果があり，膠原病性肺高血圧症に対する免疫抑制療法は，現時点で必ずしも推奨される治療法ではないが，試みる価値はあると報告している．

活動性のない膠原病性肺高血圧症の治療では，まず一般的なPAHの治療が基本となる．

図1は現在のPAHの治療法をまとめたものである．イマチニブは肺線維症に伴う肺高血圧症で有効とする報告もある．

1）M. Kataoka, T. Sato, H. Yoshino：杏林大学医学部循環器内科 2）T. Kawakami, Y. Tamura：慶應義塾大学医学部循環器内科

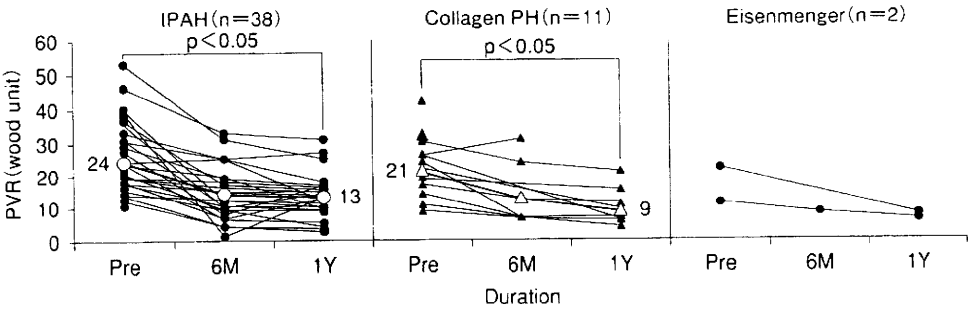

図2　慶應義塾大学病院におけるエポプロステノールの効果

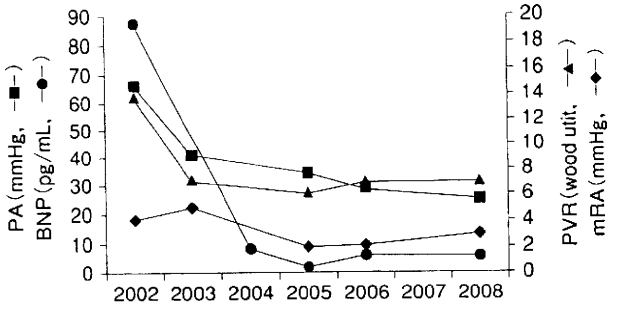

図3　臨床経過

PAH治療薬の効果

1．エポプロステノール

1）PVRに対する効果

図2は当院において，2009年春までにエポプロステノールを投与した症例のPVRを調べた結果である．エポプロステノール投与前と投与1年後の比較において，IPAH，膠原病性肺高血圧症ともにエポプロステノール投与によってPVRが有意($p<0.05$)に低下した．エポプロステノールはIPAHのみならず，膠原病性肺高血圧症にも有効であることが確認された．

2）症例報告

症例は37歳，女性．主訴は息切れである．SLEに伴う肺高血圧症で，PA圧が106 mmHg，PVRは14 wood unitであった．エポプロステノール投与に伴い，BNP値が著明に低下し，PA圧，肺血管抵抗，mRAともに低下した(図3)．

2．ボセンタン

ボセンタンに関しては，BREATHE-I[2]の結果がある．PAH症例213例をプラセボ群，ボセンタン投与群(125 mg, 250 mg)の3群に割り付けたプラセボ対照二重盲検比較試験である．膠原病性肺高血圧症症例が22%含まれた集団で，ボセンタン投与16週後の6分間歩行距離がそれぞれ−7.8 m，26.8 m，46.5 mとプラセボと比較してボセンタン投与群で有意な延長が認められた．

ボセンタンの6カ月間単独投与について検討したGirgisら[3]の報告では，IPAHより膠原病性肺高血圧症で有効性が低いものと思われる．

自験例では，ボセンタン投与によるPVR改善効果はIPAH症例で有意であった($p<0.05$)．膠原病性肺高血圧症は3例と少ないこともあって改善傾向は明確でなく，BREATHE-Iの結果と同様であった(図4)．

3．シルデナフィル

Badeschら[4]は，膠原病性肺高血圧症症例に対し，シルデナフィルがベースラインからの歩行距離を有意に延長したことを報告している．一方で，Mathaiら[5]の報告では，ボセンタンにシルデナフィルを追加した場合，IPAH症例では13例中5例で平均6分間歩行距離が延長したのに対し，膠原病性肺高血圧症で延長がみられたのは12例中2例であった．ボセンタン，シルデナフィルなどの経口薬は，IPAHでより有効性が高いことが示された．

自験例でも，シルデナフィル投与によりIPAHのPVRが有意に改善しているのに対して，膠原病性肺高血圧症では明確な改善は認められず，過去の報告と一致する結果となった(図5)．

予後

膠原病性肺高血圧症は，IPAHと比較して予後不良であることは以前より指摘されていた．2000年までの報告[6]では，膠原病性肺高血圧症は2年生存率が20%程度で，2000年から2005年の報告[7]でも，IPAHと比較して予後不良である(図6)．

自験例でも，膠原病性肺高血圧症症例の生存率は，IPAHと比較して予後不良であった．桑名ら[8]も，NYHAⅢ度，NYHAⅣ度のIPAH症例ならびに膠原病

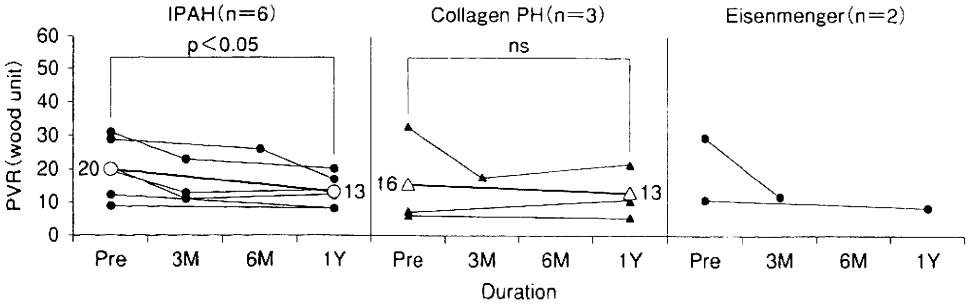

図4　慶應義塾大学病院におけるボセンタンの効果

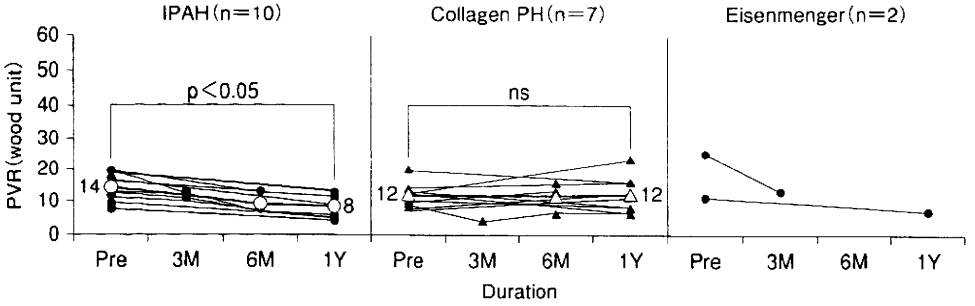

図5　慶應義塾大学病院におけるシルデナフィルの効果

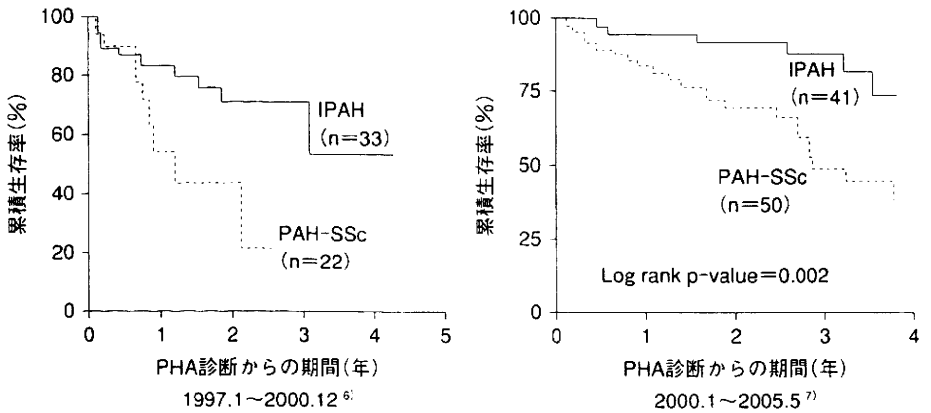

図6　PAH-SScとIPAHの予後の比較

性肺高血圧症症例にエポプロステノールを投与し，膠原病性肺高血圧症41例のNYHA Ⅳ度投与開始症例の3年生存率49.8%に対し，Ⅲ度開始症例で81.1%と，従来の報告と比較してエポプロステノール投与によってはるかに改善していることを示している．

まとめ

膠原病の活動性のある肺高血圧症では，原病の治療で改善を示す場合がある．膠原病の活動性のない肺高血圧症では，肺動脈性肺高血圧症の治療が基本となる．膠原病性肺高血圧症にはエポプロステノールがより有用な可能性があり，ボセンタン，シルデナフィルは過去の報告，自験例ともにエポプロステノールほどの効果は認められなかった．

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質疑応答

座　長：佐藤　　徹（杏林大学医学部循環器内科）
松原　広己（国立病院機構岡山医療センター循環器科）
演　者：片岡　雅晴（杏林大学医学部循環器内科）

松原（座長）　ありがとうございました．ただいまの演題にコメント，質問等はありませんでしょうか．

清水（富山大学附属病院病理部）　膠原病肺の標本を見ることがあるのですが，膠原病肺は非常に複雑で，血管病変，気道病変，間質性肺炎が複雑に関与していると思うのです．その関与の程度によって治療法を変える必要はあるのでしょうか．

片岡（演者）　肺高血圧症は様々な原因があり，その関与によって治療法を変更するといった指針はありません．免疫抑制療法で改善することもあることから，膠原病とIPAHをある程度分けて考えていく必要はあると思います．

清水　先生は膠原病をひとくくりにしておられたのですが，疾患ごとに分けて考察する必要はないでしょうか．

片岡　ご指摘のとおりだと思います．私の調べた範囲では，強皮症ならPDGFが関与するなど，膠原病の中でもそのメカニズムは微妙に異なることが指摘されているので，今後十分検討していきたいと思います．

佐藤（共同演者）　ご指摘のとおり，SLE群と強皮症群で病態が異なります．症例数が少なく，両者を分離して統計学的な解析を行うには至っていませんが，SLE由来の血管炎にはエポプロステノールが有効で，強皮症に対する有効性は低い印象があります．

ただ，IPAHは非常に多様な疾患で，その平均よりは，強皮症の方が少しいい印象はもっていますが，病因別の違いなどは症例を積み重ねて今後検討していく必要があります．

京谷（京谷医院）　先生がおっしゃるように，病態によって治療効果は当然異なると思います．

国立循環器病センター在籍中に経験した症例でいいますと，佐藤先生がおっしゃったように，明らかに線維化を主体とするSSc，PMを背景として多少肺線維症を合併している症例に，エポプロステノールやボセンタンといった肺血管拡張療法を行っても予後不良です．

それに比べてSLEとか，MCTDのような症例で，膠原病の活動性が乏しく，心不全増悪因子がほとんどない症例の反応は極めて良好です．

また，以前リウマチ医のグループから肺高血圧症の予後解析が発表されました．PAH，慢性肺塞栓症など膠原病由来の様々な肺高血圧症が，検査や分析が不十分なまま検討されていました．そうした場合，効果判定が難しい症例が入ってくると思いますので，そうした点を猪熊先生にぜひご指摘いただけたらと思っています．

猪熊（日本赤十字社医療センターリウマチセンター）　様々なご専門の先生方がこの場におられますので，極めて単純化しますと，「活動性のある膠原病」ではなく，「ステロイドが効くような病態を肺の血管に現している場合は効き得る」という言い方が適切だと思います．それはSLE，あるいはSLEの因子をもったMCTDのケースといえます．

ところが，ステロイドでSLEやMCTDのコントロールに伴って肺高血圧症のコントロールも良好であったとしても，緩解が続く症例がどれだけあるか，私は疑問に思っています．

自験例でいえば，SLEやMCTDが一見良好にコントロールされたとしても，肺高血圧症が緩解に至らない症例があります．その場合，一般のSLE，MCTDに比べて，ステロイドでコントロール可能な基礎疾患の炎症に加えてプラスαの因子が肺血管にあるではないかと想定しています．その点から考えますと，肺血管障害の成立機序によって治療法を選択するべきです．ステロイドあるいは免疫抑制薬が十分有効といえる症例がどれだけあるか，疑問に思っています．

非活動性に関しても，例えばレイノー症状のあるSSc症例を思い浮かべると，レイノー症状があること自体非活動性とはいえないわけです．レイノー症状をある程度コントロールするには，おおむね3年以上かかるわけですから，非活動性という言葉は，一定の例に限ると思います．

Zac1 Is an Essential Transcription Factor for Cardiac Morphogenesis

Shinsuke Yuasa, Takeshi Onizuka, Kenichiro Shimoji, Yohei Ohno, Toshimi Kageyama, Sung Han Yoon, Toru Egashira, Tomohisa Seki, Hisayuki Hashimoto, Takahiko Nishiyama, Ruri Kaneda, Mitsushige Murata, Fumiyuki Hattori, Shinji Makino, Motoaki Sano, Satoshi Ogawa, Owen W.J. Prall, Richard P. Harvey, Keiichi Fukuda

Rationale: The transcriptional networks guiding heart development remain poorly understood, despite the identification of several essential cardiac transcription factors.

Objective: To isolate novel cardiac transcription factors, we performed gene chip analysis and found that *Zac1*, a zinc finger–type transcription factor, was strongly expressed in the developing heart. This study was designed to investigate the molecular and functional role of Zac1 as a cardiac transcription factor.

Methods and Results: Zac1 was strongly expressed in the heart from cardiac crescent stages and in the looping heart showed a chamber-restricted pattern. Zac1 stimulated luciferase reporter constructs driven by *ANF*, *BNP*, or *αMHC* promoters. Strong functional synergy was seen between Zac1 and Nkx2-5 on the *ANF* promoter, which carries adjacent Zac1 and Nkx2-5 DNA-binding sites. Zac1 directly associated with the *ANF* promoter in vitro and in vivo, and Zac1 and Nkx2-5 physically associated through zinc fingers 5 and 6 in Zac1, and the homeodomain in Nkx2-5. *Zac1* is a maternally imprinted gene and is the first such gene found to be involved in heart development. Homozygous and paternally derived heterozygous mice carrying an interruption in the *Zac1* locus showed decreased levels of chamber and myofilament genes, increased apoptotic cells, partially penetrant lethality and morphological defects including atrial and ventricular septal defects, and thin ventricular walls.

Conclusions: Zac1 plays an essential role in the cardiac gene regulatory network. Our data provide a potential mechanistic link between Zac1 in cardiogenesis and congenital heart disease manifestations associated with genetic or epigenetic defects in an imprinted gene network. (*Circ Res.* 2010;106:1083-1091.)

Key Words: heart development ■ transcription factor ■ Zac1/Plagl1

The importance of transcription factors in development and cell differentiation has recently been underscored by the discovery that the introduction of 4 transcription factors into fibroblasts produces pluripotent stem cells.[1] Heart development is known to be regulated by a number of highly conserved transcription factors, although the mechanisms and logic of that regulation remain unclear. GATA4, myocyte enhancer factor (MEF)2C, serum response factor (SRF), Tbx5, and Nkx2-5 are expressed in the heart and play essential roles in its formation.[2–5] Furthermore, many of these transcription factors interact and act cooperatively and synergistically to direct cardiac developmental programs.[6] Despite their importance in cardiac development, however, none of the factors shows heart-specific expression, and it seems unlikely that a single factor determines cardiac cell fate.

We reported previously that transient treatment of differentiating embryonic stem cells with bone morphogenetic protein antagonists, efficiently induces cardiomyocyte differentiation.[7] Exploiting this system, we subsequently screened embryonic stem cell–derived cardiomyocytes for novel cardiac transcriptional factors using a gene chip analysis and found abundant cardiac expression of the zinc finger protein gene, *Zac1*. Zac1 was initially identified as an antiproliferative protein,[8] with subsequent studies implicating Zac1 in tumor suppression and organ development.[9,10] Furthermore, *Zac1* expression is regulated epigenetically during normal development. Imprinted genes are expressed from one allele according to their parent of origin, and this phenomenon is essential for mammalian embryogenesis. *Zac1* is a paternally expressed, imprinted gene.[10] Although imprinted genes are

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Non-standard Abbreviations and Acronyms	
ANF	atrial natriuretic peptide
BNP	brain natriuretic peptide
ChIP	chromatin immunoprecipitation
E	embryonic day
EB	embryoid body
ES	embryonic stem
GST	glutathione *S*-transferase
LOT1	lost on transformation 1
MEF2C	myocyte enhancer factor 2C
MHC	myosin heavy chain
MLC	myosin light chain
P	postnatal day
PLAG	pleomorphic adenoma gene
SRF	serum response factor
ZRE	Zac1-response element

important for mammalian development, their roles in heart organogenesis are unknown.

In the present study, we investigated how Zac1 is involved in heart development. We show that Zac1 is an essential cardiac transcriptional factor, being highly expressed in mouse hearts from embryonic day (E)8.5 to adulthood in a chamber-restricted pattern. Zac1 was found to bind directly to the atrial natriuretic peptide gene (*ANF/Nppa*) promoter in vitro and in vivo, and to possess potent transcriptional activity. Nkx2-5 and Zac1 bound to adjacent sites within the *ANF* promoter, physically interacted, and synergistically activated cardiac gene expression. The *Zac1* promoter was activated by Nkx2-5 in vitro, whereas Nkx2-5–null mice showed decreased *Zac1* expression. Genetic inactivation of *Zac1* in mice (paternal-mutated heterozygote-descendent mice) induced defective embryonic heart development and reduced expression of chamber and myofilament genes. Our results indicate that Zac1 is an essential transcription factor for cardiac morphogenesis. Moreover, this is the first report that an imprinting gene mutation causes abnormal development of the heart.

Methods

Experimental procedures for in situ hybridization, animal study, immunostaining, Western blotting, plasmids, cell culture, electrophoretic mobility-shift assay, chromatin immunoprecipitation (ChIP) assay, glutathione *S*-transferase (GST) pull-down assay, RT-PCR analysis, and statistical analyses are provided in the expanded Methods section in the Online Data Supplement, available at http://circres.ahajournals.org.

Results

Zac1 Expression in the Embryonic Heart

We used gene chip analysis to search for novel cardiac transcription factors. Initially, we screened for genes upregulated in Noggin-treated differentiating embryonic stem (ES) cells that contained conserved transcription factor motifs and then confirmed the expression in the heart by whole-mount in situ hybridization. We also analyzed the transcriptional potency of each identified factor in vitro using the *ANF* promoter as target gene. The *ANF* promoter is a marker of the developing chamber myocardium, and is responsive to various signals, including those controlling cardiac growth, remodeling and pathological overload.[11] We screened for upregulated genes by comparing cardiomyocyte-rich differentiating ES cell–derived embryoid bodies (EBs) and nontreated EBs at day 6 of culture. Three hundred fifty-three genes were upregulated (>4-fold) in Noggin-treated EBs. Among them, 13 genes encoded a recognizable conserved transcription factor motif and had not yet been analyzed in the context of heart development. These were analyzed for cardiac expression, and 6 genes were analyzed for *ANF* promoter transactivation.

In situ hybridization of staged mouse embryos showed weak expression of *Zac1* in the cardiac crescent and other embryonic sites at E7.75 and stronger heart expression at E8.5, E9.0 and E9.5 (Figure 1A). Expression at E8.5 was enriched in chamber myocardium. Immunostaining revealed Zac1 protein expression in the heart at E8.5, E9.5, and E10.5, with a heart expression pattern similar to that of α-Actinin, but included more extensive expression in mesenchyme dorsal to the heart tube, corresponding to the second heart field (SHF) (Figure 1B). Zac1 protein expression was also enriched in chamber myocardium at E9.5 and E10.5, being lower in nonchamber myocardium of the atrioventricular canal (Figure 1B). In COS7 cells, overexpressed Zac1 was localized to the nucleus, as assessed by immunohistochemistry with an anti-Zac1 antibody (Figure 1C). Fractionation of COS7 cells transfected with increasing amounts of expression vector followed by SDS-PAGE and immunoblotting confirmed the specific accumulation of Zac1 in the nuclear (Figure 1D).

Zac1 Is a Potent Activator of *Nppa* Gene Expression

We used the gene promoters from *ANF*, brain natriuretic peptide (*BNP/Nppb*), and α-myosin heavy chain (*α-MHC/Myh6*) to evaluate the transactivational potency of Zac1 in COS7 cells in comparison to that of cardiac transcription factors MEF2C, GATA4, and SRF. Zac1 activated these promoters in a manner similar to the other factors (Figure 2A), in the case of *ANF* >250-fold. We also performed the luciferase assay using neonatal rat ventricular cardiomyocytes (Online Figure I). In these cells, Zac1 increased *ANF* and *BNP* promoter activities, as did the other transcription factors; however, relative transactivation was not as strong as in COS7 cells. The α-MHC promoter did not significantly respond to any of the factors, likely because cardiac transcription factors including Zac1 are strongly expressed in these cardiomyocytes and the effect of additional expression is weak or insignificant, depending on the promoter. Although Zac1 has been identified as a transcription factor and its binding sequence reported,[12] homologous sequences were not identified in the *ANF* promoter. To show that the Zac1-dependent *ANF* promoter activation was regulated in a DNA-binding–dependent manner, we constructed a series of *ANF* promoter mutants and mapped the *cis*-regulatory sequence that mediates the response to Zac1 to the region from

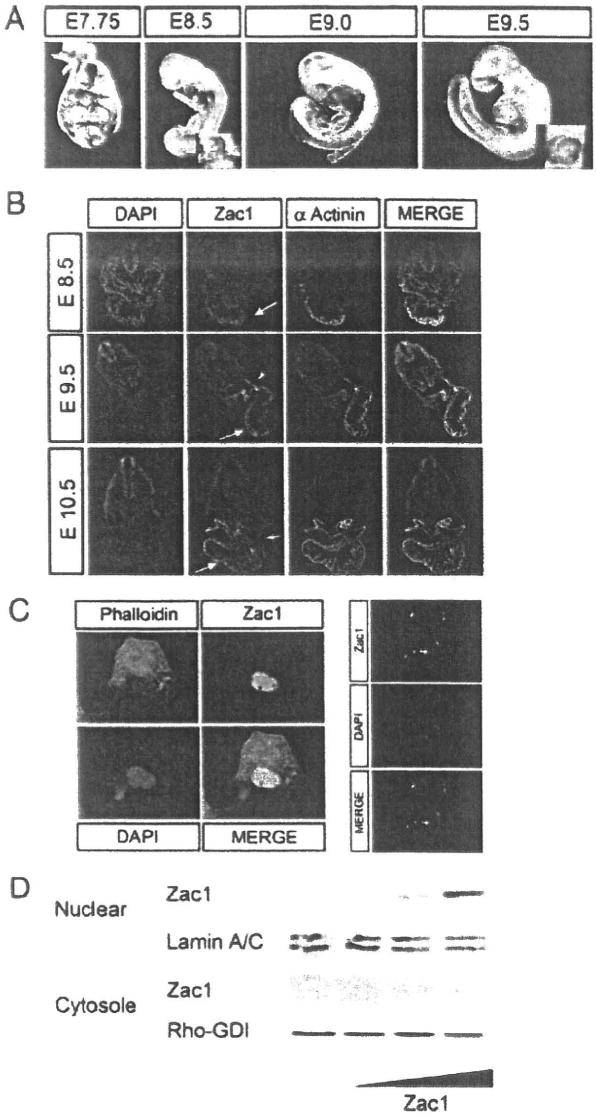

Figure 1. Expression of Zac1 in the murine embryonic heart. A, *Zac1* transcripts were detected in mouse embryos by whole-mount in situ hybridization. *Zac1* expression is weakly expressed in the cardiac crescent at E7.75 but detected throughout the heart at E8.5, E9.0, and E9.5. Frontal view of heart is shown in the **inset. B,** Immunostaining for the Zac1 protein in E8.5, E9.5, and E10.5 mouse embryos (transverse section). Zac1 protein is expressed in the heart enriched in chamber myocardium, whereas α-actinin is expressed throughout the heart and in the somites. Expression at E8.5 was enriched in chamber myocardium **(arrow)**. Zac1 expression included more extensive expression in mesenchyme dorsal to the heart tube, corresponding to the SHF **(arrowhead)**. Zac1 protein was also enriched in chamber myocardium **(arrow)** at E9.5 and E10.5, being lower in nonchamber myocardium of the atrioventricular canal **(short arrow)**. **C,** Immunostaining of Zac1 protein in transfected COS7 cells, showing expression in the nucleus. **D,** Subcellular location of Zac1 protein in transfected COS cells, as detected by Western blotting. The nuclear accumulation of Zac1 is proportional to the DNA dosage used for transfection. Lamin A/C is a nuclear protein control, and Rho-GDI is cytosolic protein control.

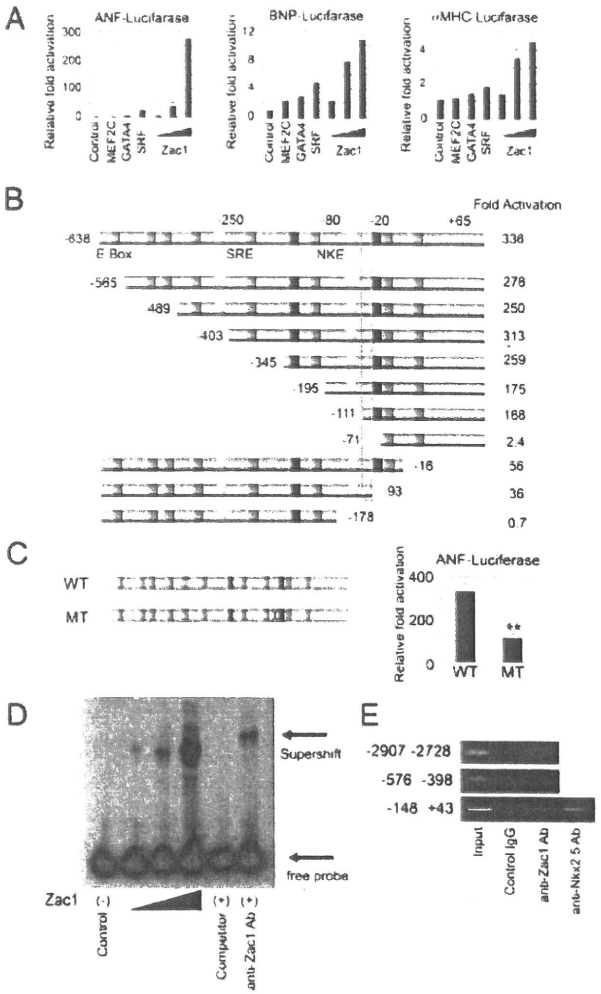

Figure 2. Zac1-transactivated ANF, BNP, and α-MHC genes. A, COS7 cells were cotransfected with a Zac1 expression plasmid and *ANF*, *BNP*, or *α-MHC*–luciferase reporter constructs. Values are expressed as the fold increase in luciferase activity compared to the empty expression plasmid (Control). **B,** COS7 cells were transfected with the Zac1 expression plasmid and the indicated *ANF* luciferase reporter constructs. Values are expressed as the fold increase in luciferase activity compared to the empty expression plasmid (Control). **Colored rectangles** indicate conserved transcription factor–binding site; **green box**, E box site; **blue box**, NKE; **yellow box**, SRF-binding element. **C,** COS7 cells were transfected with the Zac1 expression plasmid and the indicated *ANF* luciferase reporter constructs. The Zac1 response element is shown in blue (wild-type [WT]), and this element is mutated in the mutant (MT) promoter. **D,** Electrophoretic mobility-shift assay reveals the binding of Zac1 to radioactively labeled ZRE. Cold competitor interferes with the binding of Zac1 to the labeled ZRE. An antibody specific for Zac1 (anti-Zac1 Ab) supershifts the Zac1/ZRE complex. **E,** ChIP analysis reveals the binding of Zac1 and Nkx2-5 to the *ANF* promoter including the region −148 to +43 in vivo. PCR-amplified bands are apparent for the input DNA and anti-Zac1 antibody–precipitated DNA.

−111 to −93 (Figure 2B). The specific DNA sequence responsible for transactivation by Zac1 was further delineated by point mutagenesis. A Zac1-response element (ZRE) candidate sequence (GCCGCCG) within the *ANF* promoter was at least in part responsible for Zac1-dependent transactivation because mutation of this sequence to GTATATG attenuated responsiveness to Zac1 (Figure 2C). An electrophoretic mobility-shift assay was performed to determine whether Zac1 bound directly to this GCCGCCG sequence. The total amount of Zac1/DNA complex increased in proportion to the

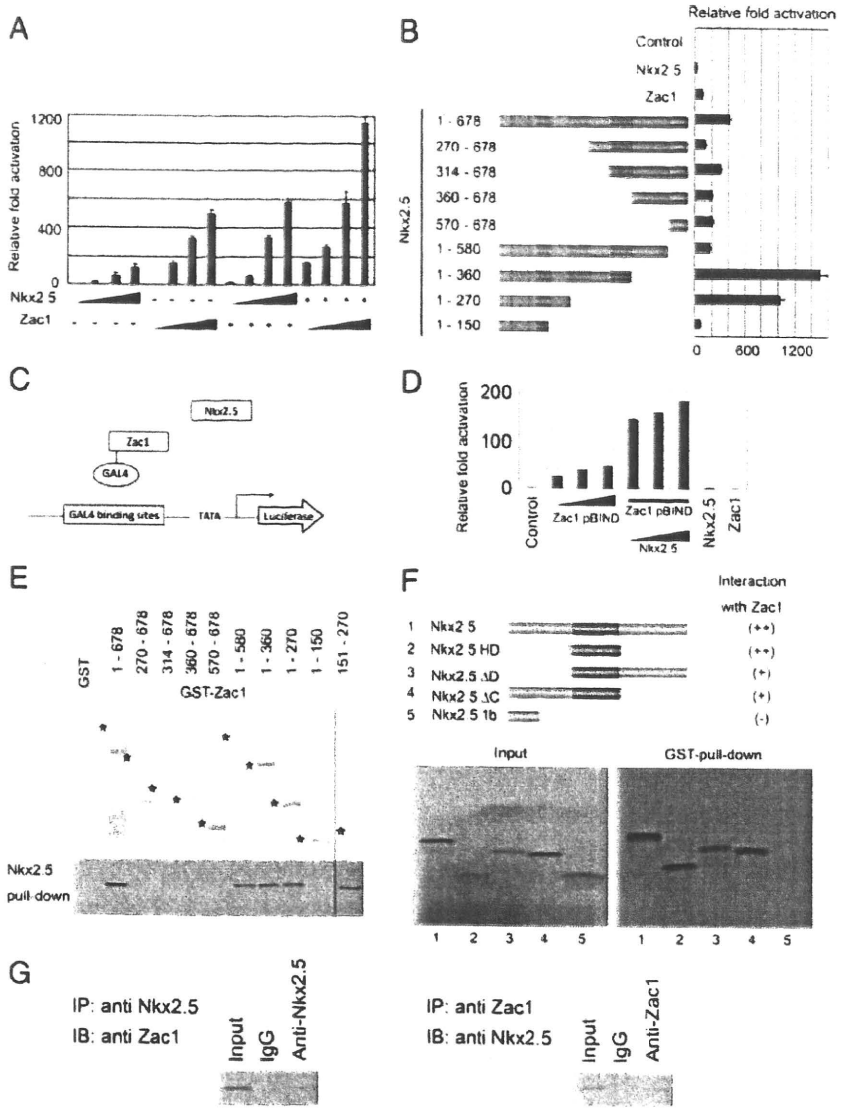

Figure 3. Zac1 and Nkx2-5 physically interact and synergistically activate *ANF* transcription. A, COS7 cells were transfected with ANF luciferase and expression vectors, encoding Nkx2-5 and Zac1. Both constructs synergistically activate *ANF* transcription (n=3). Nkx2-5 (10 to 300 ng); Zac1 (10 to 300 ng). **B,** Deletion mutants of *Zac1* were tested for their abilities to synergize with Nkx2-5 to activate *ANF* luciferase in COS7 cells. Values are expressed as fold increase in luciferase expression compared to the control. **Colored rectangles** indicate conserved protein motifs; **green box**, zinc finger motif; **blue and red boxes**, amelogenin motif; **brown box**, trypan PARP-like motif. **C,** Zac1 was fused with GAL4. A luciferase gene controlled by multiple GAL4-binding sites was used. Nkx2-5 cannot directly bind to GAL4 sites. **D,** Zac1-GAL4 increased the transactivation by DNA binding and Nkx2-5 increased this transactivation without direct DNA binding in presence of Zac1-GAL4. Wild-type Nkx2-5 and wild-type Zac1 alone did not show transactivation. **E,** GST-Zac1 deletion mutants were incubated with [^{35}S]methionine-labeled Nkx2-5 translated in vitro. The input Zac1 deletion mutant proteins are shown at **top**. Nkx2-5 proteins that bind to GST-Zac1 deletion mutants are shown at **bottom**. **F,** GST-Zac1 was incubated with [^{35}S]methionine-labeled Nkx2-5 deletion mutants translated in vitro. The input Nkx2-5 deletion mutant proteins are shown in the left panel. Nkx2-5 proteins that bind to GST-Zac1 deletion mutants are shown in the right panel. **G,** Coimmunoprecipitated proteins for Nkx2-5 or Zac1 were analyzed by immunoblotting using Zac1 or Nkx2-5 antibody. Nkx2-5 associated with Zac1 in neonatal heart extracts.

nuclear-localized Zac1 protein in COS7 cells at increasing DNA dosage. Furthermore, this complex was extinguished by the addition of cold competitor and was supershifted by the anti-Zac1 antibody (Figure 2D). To confirm that Zac1 binds to the *ANF* promoter in vivo, we used a ChIP assay. Cross-linked chromatin obtained from neonatal rat hearts was immunoprecipitated with the anti-Zac1 antibody. The precipitated chromatin DNA was then purified, and PCR analysis for enrichment of the target sequences revealed that Zac1 bound directly to the *ANF* promoter in vivo (Figure 2E). ChIP assay also showed that Nkx2-5 bound to same promoter region which includes an Nkx2-5–binding region (NKE). Zac1 did not bind to distant promoter regions which do not include a ZRE.

Zac1 Activates *ANF* Gene Expression Synergistically With Nkx2-5

The Zac1 DNA-binding site within the *ANF* promoter is adjacent to the reported binding site for Nkx2-5.[13] Therefore, we used the *ANF* promoter to ascertain whether Zac1 acts synergistically with Nkx2-5 to activate transcription. Vectors for these transcription factors were cotransfected at different DNA dosages into COS7 cells (Figure 3A). Zac1 activated the *ANF* promoter >1100-fold in a dose-dependent manner and this required the presence of Nkx2-5. Moreover, maximum activation by Nkx2-5 (>600 fold) required Zac1. To identify the protein domain of Zac1 that is involved in this synergistic activity with Nkx2-5, we cotransfected several mutated forms of Zac1 and Nkx2-5 into COS7 cells and measured the transcriptional activity of the *ANF* promoter (Figure 3B). Deletion of the 6 zinc finger domains in Zac1 (green domains in Figure 3B) reduced its ability to stimulate transcription. Notably, carboxyl-terminal deletion mutants 1 to 360 and 1 to 270, which potentially lack C-terminal repression domains, showed strong synergistic activities with Nkx2-5 (1000- to 1400-fold), which in turn was reduced by deletion of the zinc finger 5 and 6 domains (Figure 3B). Therefore, our data implicate zinc finger domains 5 and 6 of Zac1 in the functional interaction with Nkx2-5. To clarify the requirement of DNA binding for the interaction between

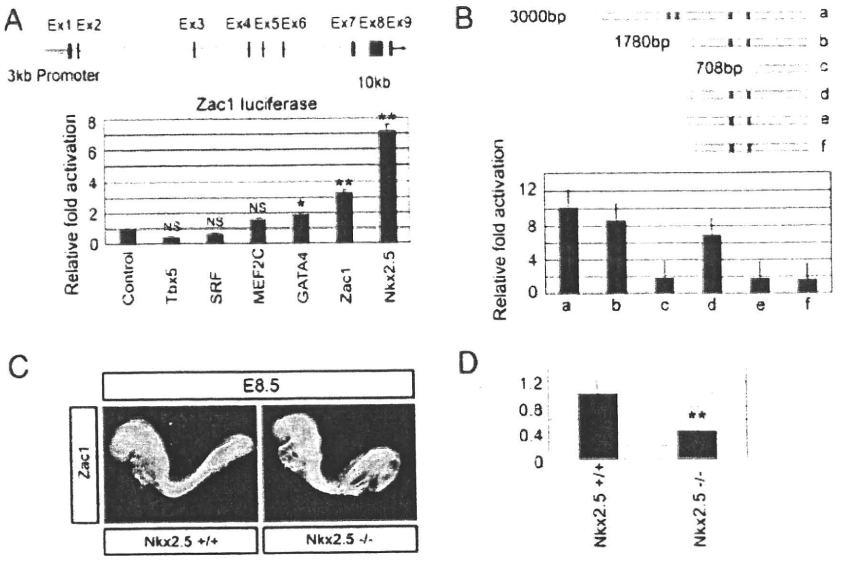

Figure 4. Nkx2-5 regulates *Zac1* gene expression. A, Structure of the mouse *Zac1* gene. The **red line** indicates the 3000-bp promoter used in this assay. COS7 cells were transfected with Zac1-luciferase and expression vectors, encoding Tbx5, SRF, MEF2C, GATA4, Zac1, and Nkx2-5. **B,** Four Nkx2-5–binding sites **(blue bar)** within the Zac1 3kb promoter/enhancer region are shown. Mutation of the third Nkx2-5–binding site **(e and f)** diminished the Nkx2-5–dependent Zac1 promoter transactivation. **C,** Detection of Zac1 transcripts by whole-mount in situ hybridization in wild-type and Nkx2-5 knockout embryos at E8.5. **D,** Quantitative RT-PCR analyses for Zac1 transcripts in wild-type and Nkx2-5$^{-/-}$ mice are shown.

Zac1 and Nkx2-5, we performed a mammalian 1-hybrid assay (Figure 3C). In this assay, Zac1, expressed as a fusion protein with the DNA-binding domain of the yeast transcription factor GAL4, was transfected with a luciferase vector under the control of multiple GAL4-binding sites (pBIND) and Nkx2-5 expression vector. Under these conditions, neither Nkx2-5 nor Zac1 could directly activate luciferase gene expression (Figure 3D). Zac1-GAL4 alone increased basal activity up to 50 fold, and Nkx2-5 increased this level of transactivation to a maximum of >200 fold (Figure 3D). These data suggest that a functional interaction between Zac1 and Nkx2-5 can occur in the absence of DNA binding.

To map the Nkx2-5–binding domain for Zac1 and to verify the physical interaction between Zac1 and Nkx2-5, GST pull-down experiments were performed using several recombinant GST-Zac1 deletion mutant fusion proteins and in vitro translated wild-type [^{35}S]methionine-labeled Nkx2-5. The wild-type Zac1-GST fusion protein interacted with Nkx2-5, as did the GST-Zac1 1 to 580, 1 to 360, 1 to 270, and 151 to 270 mutants, which encompass the zinc finger 5 and 6 domains (Figure 3E). The results indicate that these 2 zinc finger domains located within the N-terminal half of Zac1 are necessary and sufficient for association with Nkx2-5. To determine the domain of Nkx2-5 that interacts with wild-type Zac1, pull-down assays were performed with GST-conjugated full-length Zac1 and [^{35}S]methionine-labeled deletion mutants of Nkx2-5 translated in vitro. Wild-type and homeodomain-containing deletion mutants of Nkx2-5, including a homeodomain-only fragment, clearly interacted with Zac1, whereas an N-terminal fragment lacking the homeodomain did not (Figure 3F). The homeodomain of Nkx2-5 is therefore necessary and sufficient to mediate association with Zac1. These results demonstrate the importance of a protein–protein interaction between Zac1 and Nkx2-5 for gene activation in the heart. Although the Zac1 constructs amino acids 270 to 678, 314 to 278, 360 to 678, and 570 to 678 do not interact with Nkx2-5, they still show significant synergy with Nkx2-5. Because those mutants contain amino acids 570 to 678, we speculated that the 570 to 678 region of Zac1 was responsible for Zac1 dominant-active activity. Its mechanistic role is independent of a protein–protein interaction with Nkx2-5, and will be further investigated.

To demonstrate this more physiologically, we performed a coimmunoprecipitation assay to assess the existence of complexes between Nkx2-5 and Zac1 in nuclear extracts from neonatal rat hearts (Figure 3G). Coprecipitation of Zac1 with immunoprecipitated Nkx2-5, and of Nkx2-5 with immunoprecipitated Zac1, was observed.

Zac1 Is Expressed Downstream of Nkx2-5

As noted above, whole-mount in situ hybridization analysis revealed expression of *Zac1* transcripts in the cardiac crescent region in embryos at E7.5, when cardiogenic precursors are specified (Figure 1A). Shortly thereafter, Zac1 was expressed strongly in a chamber-restricted manner in the developing heart tube. To investigate the regulation of *Zac1* expression in the heart, we evaluated a 3000bp *Zac1* 5′ proximal, *cis*-regulatory fragment, which contained numerous putative cardiac transcription factor binding sites as predicted by the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html). Although Tbx5, SRF, and MEF2C had no significant effect on transcriptional activity, both Zac1 and Nkx2-5 specifically augmented *Zac1* expression (Figure 4A). The activity of Zac1 suggests autoregulation, perhaps in collaboration with Nkx2-5. To clarify the role on Nkx2-5 on *Zac1* promoter activation, we deleted or mutated several Nkx2-5–binding sites found within the 3kb promoter fragment (Figure 4B). Of the 4 consensus Nkx2-5–binding sites detected, mutation of the third site alone or in combination with other sites diminished Nkx2-5–dependent *Zac1* transactivation (Figure 4B). We also examined the expression of *Zac1* in *Nkx2-5*–null embryos to confirm that Nkx2-5 regulates *Zac1* expression in vivo. *Zac1* mRNA levels were downregulated, as assessed by whole mount in situ hybridization, and quantitative RT-PCR analysis indicated a reduction to approximately one-third of wild-type levels at E8.5 in *Nkx2-5*$^{-/-}$ embryos (Figure 4C and 4D). These results

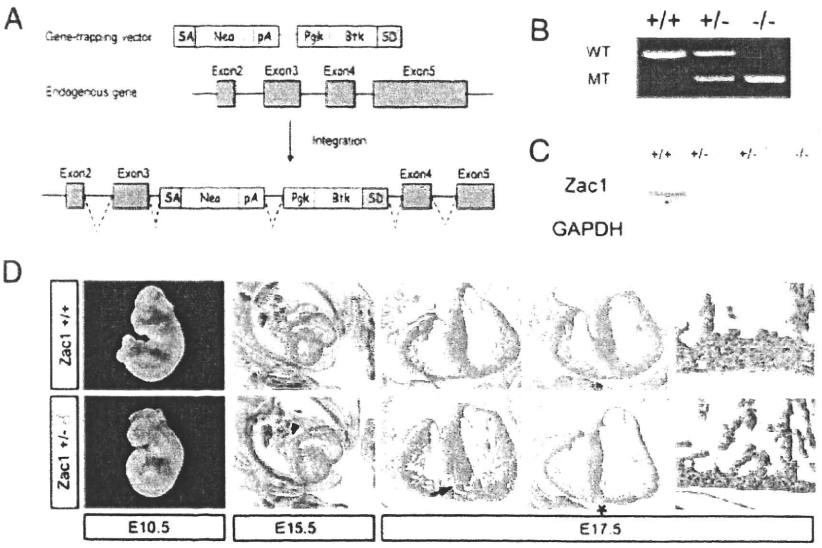

Figure 5. *Zac1* gene targeting–induced cardiac malformations. A, Schematic representation of the gene-trapping vector **(top)**, as well as wild-type **(middle)**, and interrupted Zac1 gene **(bottom)**. **B,** Genotyping by PCR. The wild-type allele yields a 407-bp product, which is absent in the homozygous mutant mice. The 250-bp product represents the targeted allele-specific band. **C,** Confirmation of Zac1 expression by Western blotting. **D,** Whole embryos at E10.5 and an embryonic heart at E15.5 and E17.5. Gross analysis of mutant embryos showed growth retardation and defective neural tube closure. Atrial septum defect **(arrowhead)** at E15.5, ventricular septum defect **(arrow)** at E17.5, and thin ventricular wall **(asterisk)** at E17.5 in Zac1-mutated embryos are shown compared to the wild-type controls.

indicate that Nkx2-5 induces and/or maintains *Zac1* expression in vivo, likely in a collaborative manner with Zac1 itself.

Partial Embryonic Lethality and Cardiac Malformation in *Zac1*-Null Embryos

To study the effect of *Zac1* mutation on mouse development, we assessed a mouse line carrying an interruption in *Zac1* generated by ES cell gene–trap methodology from Lexicon Pharmaceuticals. This mouse line contains an insertion in intron 3, which is predicted to induce a null mutation of the *Zac1* gene (Figure 5A). We confirmed the expected genomic mutation by PCR analysis (Figure 5B). Because *Zac1* shows only paternal expression, being a maternally imprinting gene, *Zac1* heterozygous animals descendent from male *Zac1* heterozygotes were indistinguishable from homozygous littermates. As we expected, Zac1 protein expression was totally abrogated in male *Zac1* mutant–descendent heterozygotes and homozygote mice (Figure 5C). Therefore, we deemed these 2 groups of mutant mice to be equivalent for the purpose of phenotypic analysis. At first we examined the gross phenotype of *Zac1* knockout mice which we generated and compared to the phenotypes previously described.[12] We confirmed that our *Zac1* knockout neonatal mice showed a similar phenotype with respect to overall weight loss, curly tail, and wrinkled skin. We analyzed 66 embryos at E10.5, 52 embryos at E15.5, and 89 embryos at E17.5. Gross examination of embryos at E10.5 revealed a defect of neural tube closure in 9% of *Zac1* mutants (Figure 5D). Histological analysis of the hearts of mutant embryos by serial sectioning along the anterior-posterior axis revealed an atrial septal defect in 42% of the mutant hearts at E15.5, as well as a ventricular septal defect involving fenestration of the muscular septum in 23% of the mutants and a thin ventricular wall in 26% at E17.5 (Figure 5D). At E17.5, we could not longer observe any of the neural tube defects evident in 9% of mutants at E10.5, suggesting a partially penetrant embryonic lethality before E17.5. Indeed, at E10.5, the expected Mendelian number of heterozygous embryos was observed (n=66). At E17.5, however, the number of heterozygous fetuses was reduced to 91% (n=89). These findings suggest neural developmental disorder as a cause of embryonic lethality in a low percentage of mutants. We also genotyped neonates at postnatal day (P)0 and P5 and adults at P90. At P0, the expected number of heterozygote mice was reduced to 91% (n=101). Although there seems to be approximately 10% reduction of heterozygous embryo, we could not obtain statistical significant differences compared to expected Mendelian ratios until P0 probably because of the limited number of embryos. At P5, this was further reduced to 44% (n=86) and at P90 was 40% (n=62), indicating an additional postnatal lethality. After P5, there are significant differences in this sample size. We did not observe any cardiac phenotypes at adult stages, suggesting that they were involved in the postnatal lethality. Varrault et al reported that approximately 30% to 50% of mutants survived to adulthood, with the percentage affected by genetic background, which is consistent with our own.[12] To confirm that the targeted locus is a null allele, we reexamined Zac1 expression in knockout mice and could not detect Zac1 by Western blot analysis using 2 different antibodies and quantitative RT-PCR analysis using independent primer sets and probes (data not shown).

The *Zac1* Mutant Mouse Shows Abnormal Cardiac Gene Expression and Patterning and a Significantly Increased Number of Apoptotic Cells in the Heart

Because *Zac1* mutant mice showed cardiac morphogenetic abnormalities, we examined the expression patterns of several cardiac genes in these mice. The expression patterns of cardiac-expressed transcription factors Nkx2-5 and GATA4 were unaffected (Figure 6A and 6B). By contrast, the expression levels of the cardiac-specific genes *ANF*, *MLC2v* (myosin light chain 2v), and *MLC2a* were significantly downregulated by both in situ hybridization and quantitative PCR (Figure 6C through 6E).

To clarify the mechanisms of cardiac malformation, we analyzed proliferation and apoptosis in the embryonic hearts. We found that *Zac1* mutant mice displayed a significantly

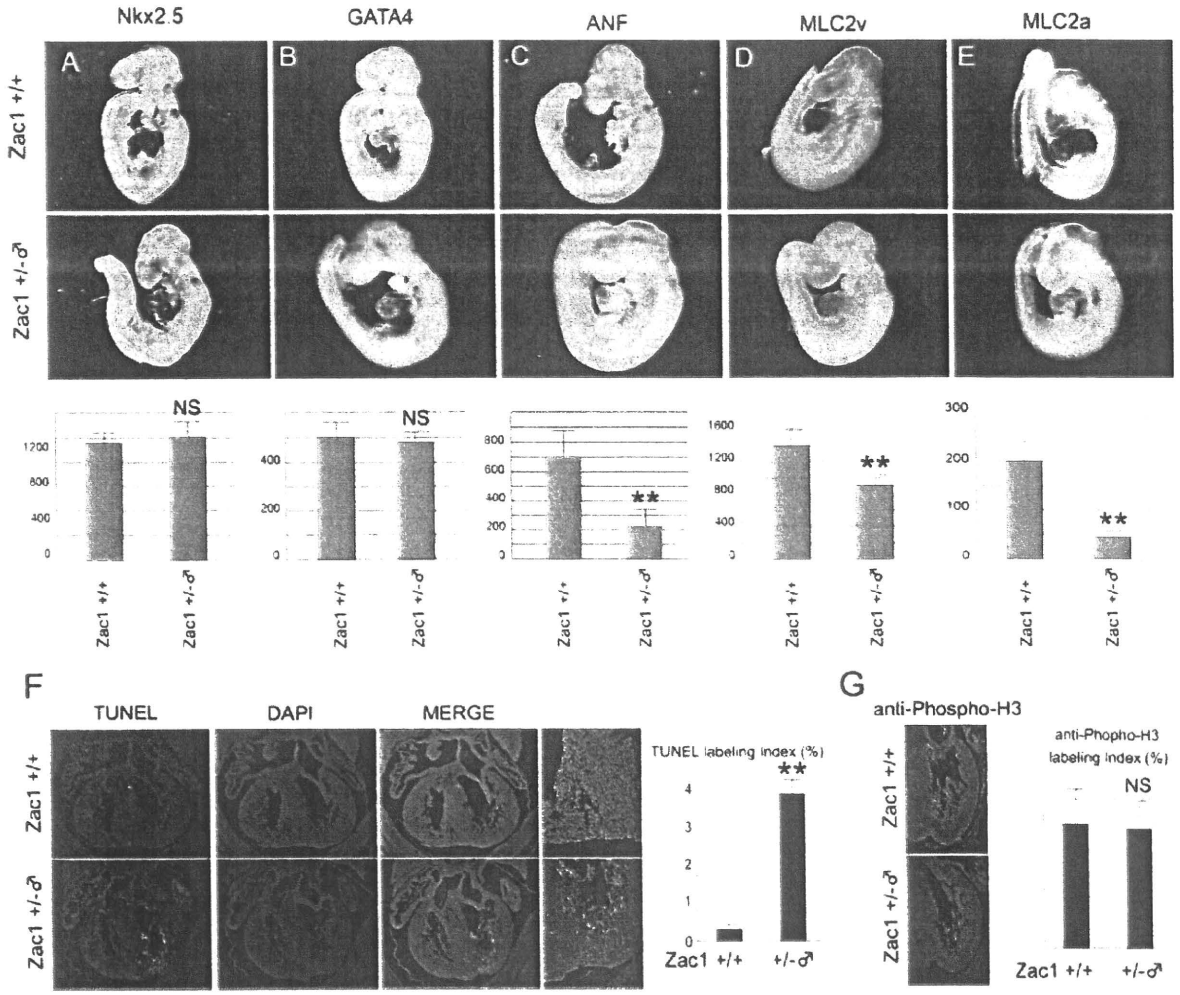

Figure 6. Altered gene expression in Zac1-mutated hearts. A and B, Expression levels of Nkx2-5 and GATA4 are normal in the *Zac1*-mutated heart, as assessed by whole-mount in situ hybridization and quantitative RT-PCR analysis. **C through E,** Expression levels of *ANF*, *MLC-2v*, and *MLC-2a* are decreased in the *Zac1*-mutated heart, as assessed by whole-mount in situ hybridization and quantitative R T-PCR analysis. **F,** Representative histological sections from the wild-type and *Zac1*-mutated hearts at E13.5 stained in the TUNEL assay. The numbers of positive cells in 5 different hearts of each genotype are shown. **G,** Representative histological sections from the wild-type and *Zac1*-mutated hearts at E13.5 stained with anti–phospho-histone H3 antibody. The numbers of positive cells in each 5 different hearts are shown.

increased number of apoptotic cells in the heart (Figure 6F). No such differences were observed in the number of proliferating cardiac cells (Figure 6G). *Zac1* is a known tumor suppressor gene, is frequently lost in multiple carcinomas, and promotes cell cycle and apoptosis.[9-14] However, many of those studies are performed in cancer cell, and there is no study in the heart. Therefore, we considered that Zac1 may have different, unique, and possibly opposite roles in cardiac development.

Discussion

In the present study, we identified the transcription factor Zac1 as an important to heart development. Initially, we used gene chip analysis of ES cell–derived cardiomyocytes to discover new cardiac-specific transcription factors.[7] Upregulated genes were tested for cardiac-specific expression and transcriptional potency using the *ANF* promoter, well studied as a cardiac target gene reflective of development and pathological hypertrophy. We confirmed Zac1 to be a strong transcriptional activator of cardiac gene in synergy with Nkx2-5 and that Zac1 itself is regulated by Nkx2-5. Analysis of a *Zac1* mutant mice verified that Zac1 is required for proper cardiac morphological development and gene expression.

The Zac1 Family of Transcription Factors

Zac1/LOT1/PLAGL1 is a member of the subfamily of PLAG (pleiomorphic adenoma gene) transcriptional factors. The PLAG family genes were defined by the capacity of PLAG1 overexpression to induce pleomorphic adenomas.[15] The PLAG family comprises *PLAG1*, *Zac1/LOT1/PLAGL1*, and *PLAGL2*. These factors share high levels of homology, especially in their zinc finger amino-terminal regions, although they are functionally distinct. *PLAG1* is a protooncogene and a target of chromosomal rearrangements that results in tumorigenesis. *PLAGL2* is induced in human acute my-

eloid leukemia, and may in fact induce acute myeloid leukemia in cooperation with other fusion genes.[16] PLAG1 and PLAGL2, therefore, have similar capabilities in tumorigenesis and have indistinguishable DNA-binding specificities, which are different from that of Zac1.[17] *Zac1/LOT1/PLAGL1* is lost in malignantly transformed rat ovarian surface epithelial cells, hence the name *LOT1* (lost on transformation).[14] However, Zac1 was also shown to regulate apoptosis and the cell cycle, accordingly named *Zac1*.[8] Subsequently, the gene symbol for this family member was designated as *PLAGL1*. Although having a similar protein structure, Zac1 appears to have an opposite function to PLAG1 and PLAGL2 in tumor formation and binds different DNA sequences.[17] Therefore, we speculated that there is no functional overlap between *Zac1* and the other *PLAG* family genes.

Imprinting Genes in the Heart

From a metaanalysis of microarray data, *Zac1* was found to be a member of an imprinted gene network.[12] Classically, both alleles of a gene were thought to be actively transcribed and functionally equivalent. Since the identification of the first autosomally imprinted genes in 1990s, researchers have tried to elucidate imprinting functions.[18] In the murine genome, approximately 600 genes are potentially imprinted,[19] and several theories have been proposed to explain why so many genes should be imprinted.[20] The ovarian time bomb hypothesis states that imprinting occurs to prevent parthenogenesis from unfertilized oocytes, which can lead to malignant trophoblastic disease.[21] Epigenetic abnormalities in imprinted regions have been implicated in a number of developmental disorders and carcinogenesis in mice and humans.[22,23]

The maternally methylated CpG island of the murine and human *Zac1* locus was identified in a screen for imprinted genes.[24,25] Genetic and epigenetic defects in the *Zac1* locus are also associated with Beckwith–Wiedemann syndrome.[26] Although Beckwith–Wiedemann syndrome is generally characterized by exomphalos, macroglossia, and giantism, cardiac manifestations are also known to occur, including congenital heart disease (ventricular septum defect, atrial septum defect, aortic stenosis) and cardiomyopathy.[27–31] Beckwith–Wiedemann syndrome is associated with a region of chromosome 11 in which many candidate disease genes are present including IGF-1 and $p57^{kip2}$. Although the molecular mechanisms underlying cardiac abnormalities seen in Beckwith–Wiedemann syndrome remain unknown, we have shown here a possible mechanistic link between *Zac1* and heart disease seen in the syndrome.

Regulation of Cardiac Gene Expression by Zac1

Our data show that Zac1 acts as a transcriptional activator for cardiac genes based on the following observations: (1) in development, Zac1 was highly expressed in the heart and enriched in chamber myocardium; (2) Zac1 bound directly to the *ANF* promoter and strongly activated the *ANF*, *BNP*, and *α-MHC* promoters; (3) Zac1 physically interacted with Nkx2-5 to synergistically activate cardiac gene expression; (4) Zac1 functioned as a downstream target of Nkx2-5 both in vitro and in vivo; (5) *Zac1* mutant mice showed cardiac gene expression abnormalities; and (6) *Zac1* mutant mice exhibited cardiac malformations.

A number of cardiac transcriptional factors collaborate in a complex manner to guide development and homeostasis in the heart. Nkx2-5, GATA4, Tbx5, MEF2C, and SRF are essential and potent cardiac transcriptional factors, regulating the expression of one another and serving to stabilize and reinforce the cardiac gene regulatory network. Zac1 expression was first observed at early stages of heart development, coincident with just after cardiac specification and expression of early transcription factors such as Nkx2-5. Our data also indicate that Nkx2-5 directly activates *Zac1* expression in the heart. We speculate that Zac1 and Nkx2-5 orchestrate and support the expression of other transcription factors and cofactors. In particular, cardiac transcription factors and Zac1 function together to stabilize the transcriptional machinery, in part by binding to adjacent sites within the promoter/enhancer regions of cardiac genes and also through direct protein–protein interaction. This robust transcriptional activation network promotes development and maturation of the heart. Our work establishes Zac1 as a new player in this network. Zac1 may provide a valuable entry point for genetic analysis heart growth and control of apoptosis and how these processes are controlled by the core, conserved transcription factor network.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Cardiac development is stringently regulated by various cardiac transcription factors, although many aspects of the underlying mechanisms remain to be elucidated.
- Mammals have evolved the intriguing process of gene imprinting, but it is not clear what roles gene imprinting plays in heart development and homeostasis.

What New Information Does This Article Contribute?

- We identify the maternally imprinted zinc finger–type transcription factor *Zac1* as a potent cardiac transcriptional activator.
- Our examination of homozygous and paternally derived heterozygous mice reveals several congenital cardiac malformations, indicating that *Zac1* is an essential transcription factor for cardiac morphogenesis.

Transcription factors play central roles in gene expression, organ morphogenesis, and pathogenesis. Although several essential cardiac transcription factors have been identified, the complex transcriptional networks in the heart are still poorly understood. To identify novel and potent cardiac transcription factors, we performed gene chip analysis using cardiomyocytes that were differentiated from ES cells. We found that the *Zac1* gene, which encodes a zinc finger–type transcription factor and is a maternally imprinted gene, was strongly expressed in the mouse embryonic heart. Zac1 is a potent transcriptional activator of several cardiac genes and binds directly to the ANF promoter. Binding sites for Zac1 within the ANF promoter were also determined. Zac1 was found to exert strong synergistic transcriptional activity and to interact physically with Nkx2-5. Nkx2-5 also activated the *Zac1* promoter, and Nkx2-5–null hearts showed decreased Zac1 expression. *Zac1*-mutated mice showed decreased levels of several cardiac-specific genes and increased numbers of apoptotic cells in the embryonic heart. The *Zac1*-mutated mice also exhibited severe cardiac deformities: an atrial septum defect, a ventricular septum defect, and thinning of the ventricular wall. Our results suggest a potential mechanistic link between genetic or epigenetic defects and congenital heart disease manifestations.

Supplement Material

In Situ Hybridization

Whole-mount *in situ* hybridization was carried out as previously described [1]. Digoxigenin-labeled RNA probes were prepared by *in vitro* transcription. The full-length cDNA for murine *Zac1* (accession no. AK142210) was obtained by RT-PCR and subcloned into the pBluescript plasmid. The cDNAs for murine Nkx2-5, GATA4, ANP, MLC2-v, and MLC-2a were kindly provided by Dr. E.N. Olson and Dr. H. Yamagishi. The probes were transcribed with T3 or T7 RNA polymerase.

Animal study

Pregnant ICR wild-type mice were purchased from Japan CLEA. All experiments were approved by the Keio University Ethics Committee for Animal Experiments.

Immunostaining

Antibodies directed against Zac1 (G-18; Santa Cruz Biotechnology, Santa Cruz, CA), actinin (EA-53; Sigma, St. Louis, MO), Lamin A/C (#2032, Cell Signaling Technology), Rho-GDI (610255, BD Biosciences), phospho-histone H3 (9071; Cell Signaling) and phalloidin (Molecular Probes, Eugene, OR) were added to the sections, followed by overnight incubation at 4°C. Next, three 5-min washes in PBS were carried out, followed by the addition of secondary antibodies conjugated with Alexa 546 (Molecular Probes), and incubation for 1 h at room temperature. The sections were washed three times in PBS for 5 min each and then observed by confocal laser-scanning microscopy (LSM510; Carl Zeiss, Jena, Germany). The TUNEL assay was performed using the ApopTag Red In Situ Apoptosis Detection kit (Chemicon International) according to the manufacturer's protocol.

Western blotting

COS7 cells were transfected with pcDNA3.1 Zac1 using Lipofectamine (Invitrogen, Carlsbad, CA). Cell extracts were isolated 24 h after transfection and separated into nuclear and cytosolic fractions. Fractionated protein lysates were resolved by SDS-PAGE and transferred to a PVDF membrane, followed by immunoblotting with rabbit anti-Zac1 antibody (Santa Cruz Biotechnology) at a dilution of 1:1,000 and horseradish peroxidase-conjugated anti-goat IgG, followed by development with the SuperSignal West Pico Chemiluminescent reagent (Pierce, Rockford, IL).

IP-western blot analysis

Total cell lysate was prepared from neonatal mouse hearts. IP-western blot analysis was performed essentially as described previously using anti-Zac1 and anti-Nkx2-5 for hearts lysate.

Plasmids

The Zac1-expressing plasmids were generated through conventional or PCR-based cloning. Deletion mutants were constructed by PCR-based mutagenesis and subcloning of the DNA fragments into the pcDNA3.1 expression vector. Site-directed mutagenesis was performed using the QuickChange kit (Stratagene, La Jolla, CA). The reporter plasmids (ANP-luciferase, BNP-luciferase, and α-MHC-luciferase) were kindly provided from Dr. E.N. Olson. The *Zac1* promoter was cloned using PCR-based techniques from a BAC clone into the pGL3 basic vector (Promega, Madison, WI). For mammalian hybrid assay, pBIND vector and pG5luc vector were purchased from Promega.

Cell culture, transfection, and luciferase assay

COS-7 cells plated in DMEM with 10% FBS were transfected with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Unless otherwise indicated, 100ng of reporter and 100ng of each activator plasmid were used. The DNA doses represented by the ramp symbol indicate 0, 30, 100 and 300ng of plasmid. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. CMV-*Renilla* luciferase was used as an internal control, to normalize for variations in transfection efficiency. All the proteins were expressed at very similar levels, as confirmed by Western blotting.

EMSA

Nuclear extracts were collected from COS7 cells that overexpressed Zac1. Double-stranded oligonucleotides for the Zac1-binding sequence '(5 -GCATCTTCTGCTGGCCGCCG-3') were synthesized, and the two complementary oligonucleotides were annealed and labeled with [α-^{32}P]-dATP using the Klenow enzyme. Labeled probes were incubated with 5 ml of nuclear extracts and 2mg of poly(dI-dC) in 20 ml of binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, 0.05% Nonidet P-40] for 30 min at room temperature. The protein/DNA mixture was resolved on a 5% polyacrylamide gel in 0.5 Tris borate/EDTA buffer at 4°C for 2 h at 150 V.

ChIP assay

For the *in vivo* ChIP experiments, extracts were prepared from five neonatal rat wild-type hearts for independent experiments. For the ChIP assays, we used the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Lake Placid, NY) and followed the instructions of the supplier. Primer in PCR reactions is 5'-ACAAGCTTCGCTGGACTGAT-3' and 5'-TCTCGGCTCACTCTCTGGTT-3' (-148 +43), 5'-CCTGACTGCTAACAGGGACA-3' and 5'-

TGTCAGGGGCTCCAAATAAG-3' (-576 -398), 5'-GAGAGGAGCTGGACCATGAG-3' and 5'-TTGAAAGCGTGAGGACTTGA-3' (-2907 -2728). The amplified region corresponded to the rat ANP promoter, which encompasses the Zac1-binding sites.

Glutathione S-transferase (GST) pulldown assay

Murine Zac1 cDNA and several DNA fragments encoding Zac1 were subcloned into the pGex-6P vector (Amersham Biosciences). GST fusion proteins were isolated by standard procedures. The plasmids that contained the deletion mutants of Nkx2-5 were gifted by Dr. I. Komuro. Proteins translated *in vitro* were labeled with [^{35}S]-methionine in the coupled transcription-translation T7 reticulocyte lysate system (Promega), and assayed for binding to the GST-fusion proteins.

RT-PCR and real-time quantitative PCR

Total RNA was extracted using the Trizol reagent (Invitrogen), and RT-PCR was performed as described previously. At least five replicates were processed for each assay. *GAPDH* was used as an internal control. For quantitative analysis of *Nkx2-5*, *GATA4*, *ANP*, *MLC2v*, and *MLC2a* expression, the respective cDNA was used as the template in a TaqMan real-time PCR assay using the ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. All samples were run in triplicate. The data were normalized to *GAPDH* expression. The primers and TaqMan probe for *Nkx2-5*, *GATA4*, *ANP*, *MLC2v*, *MLC2a* and *Zac1* were Mm00657783_m1, Mm00484689_m1, Mm01255747_g1, Mm00440384_m1, Mm00491655_m1, and Mm00494251_m1, respectively.

Generation of mutant mice

The *Zac1*-mutated mice were generated by Lexicon Pharmaceuticals from ES cells that corresponded to OST181461 (OmniBank sequence tag) and that were targeted by gene trapping. The gene-trapping vector contained a retroviral 5 -end long terminal repeat (LTR), a splice acceptor sequence, neomycin gene (Neo), and partial first intron of the murine *Bruton's tyrosine kinase* (*Btk*) gene as the 3 -trapping component, rather than a selectable marker, which was regulated by the 3-phosphoglycerate kinase 1 (PGK-1) gene promoter, a splice donor sequence, and a 3'-LTR[2]. Retroviral infection, selection, and screening of the ES cells were performed as previously described. The gene-trapping vector was inserted at the third intron of the *Zac1* gene (corresponding to OST181461) in the ES cells, as detected by inverse-PCR. ES cells were selected for blastocyst injection into C57BL/6 mice to produce chimeric mice. Heterozygous and homozygous animals were analyzed along with littermate control animals.

Statistical Analyses

Values are presented as mean ± SEM. Statistical significance was evaluated with the unpaired Student *t* test for comparisons between 2 mean values. A chi squared analysis for comparisons between 2 groups. Comparisons between >3 groups were performed with ANOVA. A value of $P<0.05$ was considered significant. *$p<0.05$, **$P<0.01$, NS; not significant.