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血管病モデルマウスと血管新生可視化マウスの資源化

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厚生科学研究費補助金　（創薬基盤推進研究事業：生物資源・創薬モデル動物研究）
総合研究報告書

血管病モデルマウスと血管新生可視化マウスの資源化

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研究要旨　血管病の研究に資するマウスとして新規に血管病関連モデルマウスと血管可視化マウスを作製し、医薬基盤研究所実験動物研究資源バンクに登録し、国内外に円滑に提供できるシステムを構築することが目標である。血管病関連モデルマウスと血管可視化マウスとして、血小板減少性紫斑病の原因遺伝子ADAMTS13の遺伝子改変マウス、。Ndrg4（N-myc downstream-regulated gene 4）遺伝子欠損マウスを作製、またFoxO、CD82強制発現マウスを作製した。複数のVEcadherin-Creマウスも樹立した。バンク機能としては開始後１００種類以上の登録を行い、実際海外にも提供できるシステムを確立した。

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A. 研究目的

国民病といっても過言ではない動脈硬化症、糖尿病、虚血性心疾患の臓器障害は、血管の生理的機能の破綻と考えられる。したがって、これらの血管病ともいえる状態の血管障害病態モデルを動物で作製し、新規薬剤の個体での薬効評価系として資することは今後の治療開発戦略として非常に重要である。特に血管可視化マウスでは、癌では血管新生の抑制効果の判定に、虚血性病変では血管新生促進効果を示す薬剤の判定に用いることも可能になる。本研究では、遺伝子改変マウスを作製し、これを資源化することを目指した。

血管病変の基礎的病態として血栓症が重要視されている。血栓症は国民の健康の大きな不安要因であり、安心・安全な生活を送るため、血栓症制圧に向けた研究が進められている。なかでも脳虚血障害は大きな社会問題であり、医学研究上の最重要課題の一つである。このため、血栓症の成因に関係する遺伝子の改変動物を用いて血栓症モデル動物を作製することを目的とした。

また血管新生は癌・虚血性心疾患では治療の標的となっている。血管新生を可視化できるマウスは、治療効果の判定に有用であると考えて血管新生可視化マウスの作製を試みる。

血管病モデルマウス、血管新生可視化マウスを国内外の研究機関へ円滑に提供するシステムとして医薬基盤研究所に実験動物研究資源バンクを設立する。この仕組みのなかに、動物を提供して、マウス等を医薬基盤研究所実験動物バンクにおいて生物資源として収集し、保存、品質管理を行い、研究者に迅速に安定的に供給する体制を構築することを目的とする。生物資源の提供が新規治療薬や予防法の開発に繋がり、国民の健康、福祉の一層の向上に寄与することうを期待した研究である。

B. 研究方法

血管内皮細胞特異的FoxO1発現マウス

FoxO1は、インスリン受容体の下流で機能する寿命調節転写因子と考えられている。また、MnSODの転写を亢進することから、血管内皮細胞にどのように作用するのか不明の分子である。Rosa26にFoxO1 3A 変異（Aktによって燐酸化されるSer，Thrを　Alaに置換してAkt活性化による核外移行を阻害された変異体）をもつcDNAを挿入し、Cre-loxPによりCreが機能したときにのみ、ROSA26によりFoxO1-3Aが発現するマウスを先ず作成した。このマウスと血管内皮細胞特異的にCre recombinaseを発現するTie2-Creマウスを交配させて、血管内皮細胞特異的にROSA26によってFoxO1-3Aを発現するマウスを得た。

Ephexin1, Ephexin5 double KOマウスの作製

チロシンキナーゼEphA4受容体の下流でRhoファミリー分子を活性化するEphexin ファミリーのノックアウトマウスを作製した。

CD82 を血管内皮細胞特異的に発現するtransgenic mouseの作製

低酸素状態で分解が抑制されることにより発現が増加するHypoxia-inducible factor (HIF)により、転写が増加する分子の一つとして同定したCD82のoverexpressionマウスを作製した。

Adamts13 ノックアウトマウスによるAdamts13の個体での機能解明

Adamts13ノックアウトマウスは私達が作製した129型遺伝背景のものを用いた。C末端欠損ADAMTS13マウスは、B6マウスがC末端欠損ADAMTS13を発現しているので、B6マウスを129マウスに10世代掛けあわせ、遺伝背景が129

型のマウスを作製して使用した。野生型マウスは129マウスを用いた。平板型フローチャンバーを用いた血小板血栓形成能、コラーゲン-エピネフリン静注による血小板数減少モデル、塩化鉄による微小血管傷害モデルは、既報にしたがって行った。

Ndrg4欠損マウスの作製

常法により、遺伝子ターゲティング法を用いてNdrg4欠損ヘテロマウスを作製し、ヘテロマウス同士を交配させて、欠損ホモマウスを作製した。Ndrg4欠損マウスの空間学習能力を調べるために、モリスの水迷路試験を行った。また、虚血ストレスに対する脳の脆弱性を調べるため、三血管閉塞法による虚血・再灌流実験を行った

血管内皮細胞特異的enhanced green fluorescent protein (EGFP)発現マウスの作製：

これまでに、VE-cadherin promoter -Cre発現マウスとloxP-stop-loxP-EGFPレポーターマウスの交配により、VE-cadherinのpromoter依存性にEGFPを発現するマウスを作製していた。

① VE-cadherin promoter (-2.5 kb) とenhancer (1st intronの5' gawa 4kb を含む) 下流に直接EGFPをつなげてpromoter 活性を可視化できるかどうかを調べるtransgenic mouseを作製した(VEcad -2.5 P/E-EGFP)。

② VE-cadherin promoter (-2.5 kb) とenhancerを含む(first intron の5'側の 4kb)領域の下流にCreを発現するマウスを作製した。(VEcad -2.5 P/E-Creマウス)。

実験動物研究資源バンクの運営と拡充

医薬基盤研究所実験動物研究資源バンクは、創薬・疾患研究への貢献を目指した厚生労働省のバンクとして、有用なマウス系統の収集、保存、供給、情報発信、マウス資源の保護預かりなどを行っている。とくに、貴重な自然発症疾患モデルマウスの資源化を進めるとともに、保護預かりサービスなどを通じて、利用者がバンクを有効に利用することで動物実験を効率良く出来るようにアドバイスを行うなど、迅速できめ細かな対応を行い、動物資源を利用しやすい環境整備に努めた。

C. 研究結果

血管内皮細胞特異的FoxO1発現マウス

Tie2-CreもマウスとROSA26 floxed FoxO1発現マウスとの交配により血管内皮細胞特異的FoxO1発現マウスを得ることができた。血管の形態学的検討でも大血管の走行や起始異常などは観察されず、肺血管も走行以上を認めなかった。また脳血管の分枝も正常であり、FoxO1の過剰発現による効果は発生に大きな影響がないことがわかった。また、加齢にともなう顕著な変化も認めなかった。

Ephexin1 Ephexin5は血圧調節に重要

Ephexin1 KO マウスをHarvard Univ. Greenberg博士より、供与していただき我々のEphexin5 KOマウスと交配することによりEphexin1/5 ダブルKOマウスを作製した。Ephexin1, Ephexin5の単独欠損マウスでは血圧に異常を示さなかったが、ダブルKOマウスでは、野生型に比較して有意な血圧低下が観察できた。

血管内皮細胞特異的CD82発現マウスの樹立

HIF1 2をoverexpression可能な、アデノウイルスを用いてヒト臍帯静脈内皮細胞にHIF1, 2を発現させて、cDNAのmicroarray解析を行った。その結果、HIFによって発現が増加する分子としてCD82分子を同定した。この分子は、細胞膜を4回貫通する分子であることが予想される。tetaraspaninに属する分子であるが、その機能は未知である。

このために、CD82の血管内皮細胞特異的発現マウスを作製した。PCR 上は、7系統のlineを得ることができた。現在血管内皮細胞での発現を免疫組織化学的に検討している。マクロの観察では、大きな血管の構築に異常は認めていない。

Ndrg4欠損マウスの作製と個体の解析

Ndrg4欠損ヘテロ、ホモマウスとも正常に発育した。

モリスの水迷路試験による行動解析では、

Ndrg4欠損ホモマウスでは、体力的な遊泳能力は正常であるにもかかわらず、野生型に比べて逃避潜時 ($N=20$, $P<0.001$) と遊泳距離 ($N=20$, $P<0.001$) が遅延していた。また、一過性局所脳虚血による脳梗塞体積の解析では、野生型に比べ、Ndrg4欠損ホモマウスでは片側運動麻痺の亢進 ($N=10$, $P=0.001$) と脳梗塞体積の増加 ($N=10$, $P=0.006$) が観察された。

ADAMTS13欠損マウスの血栓症における機能

ADAMTS13ノックアウトマウスのVWFマルチマーは、野生型マウスのマルチマーより高分子量で検出されたが、C末端欠損マウスのマルチマーは野生型マウスと同等の分子量分布を示した。またノックアウトマウスでは、細動脈のずり速度に相当する1000 s^{-1}にて測定した*in vitro*血小板血栓形成能が亢進していたが、C末端欠損マウスでは、野生型マウスとの違いは認められなかった。

VEcad P/E-EGFP, VEcad P/E-Creマウスの樹立

VEcad P/E EGFPトランスジェニックマウス、VEcad P/E-Creトランスジェニックマウスをそれぞれ5系統、6系統をPCRで確認できた。VEcad P/E EGFPトランスジェニックマウスでは、蛍光実体顕微鏡的にEGFPを血管で検出できなかった。このため同ラインの樹立は意味がないと考えた。また、Creの発現に関しては、現在EGFP レポーターマウ

スとの交配により血管での発現を確認中である。

実験動物研究資源バンクの資源保存技術の改良

Balb/cマウスでは複数の雄を用意して運動性の良い精子を選択して体外受精することにより受精率50-70%の安定した成績を得ることが出来た。また、BALB/cマウス凍結胚の融解-移植による生体への復元率は4-13%と低かったが、凍結精子を融解して体外受精に用いたところ、受精率は20%程度と低いものの、胚移植した場合は、産仔率が最大30%を示した。このことは、バンクとしての資源保存法として、BALB/cの場合は精子凍結が有効な手段となる可能性が示された。

医薬基盤研究所実験動物研究資源バンクは、疾患研究・創薬研究用疾患モデルバンクとして整備を引き続き進め、今年度新規に血管病モデル関連マウス3系統を含む27系統を分譲可能とし、総公開系統数が121系統に達し、利用者数が順調に増大し事業の拡大を行った。今年度は、とくに新規に血管病関連モデルマウス5系統の遺伝子導入マウスの作製を順調に開始し、血管病研究への貢献が期待される。

D. 考察

FoxO1はstress responseで核内で機能する。したがって恒常的に核内で発現をさせたときに、加齢とともに様々な細胞外刺激で核外移行ができなくなっていることにより生じる病態があると考える。したがって、今後加齢、動脈硬化症モデルとの交配によりさらに血管での機能について検討が必要である。

低酸素による血管新生の病態を検討するマウスとしてCD82を血管内皮細胞で発現するマウスを作製した。HIFによってCD82は発現が増加することを認めたのでCD82の血管内皮細胞特異的発現マウスを樹立することによりCD82の機能を検討することを計画した。転写因子HIFは癌、あるいは虚血によって誘導される血管新生において不可欠な転写因子であり、この転写因子によって誘導される分子が如何にして血管新生を調節するかを理解しようとした。Vascular endothelial grwoth factor （VEGF） 以外にもHIFによって転写活性が亢進する分子は多数同定されてきており、CD82も今後低酸素による網膜血管病変における機能などを検討する必要があると考えた。

血管特異的にEGFPを発現するマウスは、血管新生のイメージングに有用であると考え、本研究を開始した。VEcad-Creマウスにより、血管を明瞭にイメージングすることはできたが、Cre/loxP システムを使用すると、一旦VEcad promoterが活性化した細胞では、絶えずCAG promoterによってEGFPが発現する。したがって、血管から他の細胞に変わった細胞でもEGFPを発現してしまう可能性は否定できなかった。最終年度にVEcad E/Pに直接EGFPをつないでVEcadの転写活性を可視化できるマウスを作製することを試みた。しかし、蛍光の発現が不十分（顕微鏡観察をするのに）であり本マウスの繁殖・樹立は断念した。

遺伝性末梢神経変性疾患

Charcot-Marie-Tooth病　４D型の疾患モデルマウスとして、*Ndrg1*ノックアウトマウスを医薬基盤研究所に寄託した。また、先天性ADAMTS13遺伝子欠損症の疾患モデルマウスとして、*Adamts13*ノックアウトマウスを医薬基盤研究所に寄託した。これらの資源化されたマウスは医薬基盤研究所を通して、希望する研究者に分譲され、創薬・疾患研究に貢献することが期待される。

NDRG4は脳および心臓で特異的に発現し、細胞障害性刺激によって発現誘導される。、Ndrg4欠損マウスを作製し、脳虚血に対する脳保護効果について評価を行ったところ、Ndrg4欠損ホモマウスは野生型に比べてまた片側運動麻痺の亢進と一致して脳梗塞体積が増加していた。

三血管閉塞法による脳虚血モデルでは、血管内の内皮細胞は無傷で保たれるため、血管内に血栓が形成されない。このため、神経細胞、グリア系細胞と血管系細胞からなる機能的ユニットの虚血に対する脆弱性を評価できると考えられる。つまり、Ndrg4欠損により脳梗塞巣体積が増加することは、Ndrg4欠損により脳保護作用が低下していると理解される。したがって、脳虚血障害の悪化を示すモデルマウスを資源化できることになった。

医薬基盤研究所実験動物研究資源バンクは、疾患研究・創薬研究用疾患モデルバンクとして整備を引き続き進め、今年度新規に血管病モデル関連マウス27系統を分譲可能とし、総公開系統数が121系統に達する規模拡張ができた。、利用者数が順調に増大し事業の拡大を行った。

基盤研実験動物研究資源バンク事業は、安定した技術と迅速、きめ細かで効率良いサービスに対する信頼が得られつつあり、今年度、利用者の飛躍的な増大に繋がったものと考えられる。さらに安定的な運営と拡充により、研究者がより利用しやすいバンクを構築するとともに、独自の血管病モデルマウスなどの資源化を進めることで、血管病の研究を始めとして、疾患研究・治療薬開発などが進展するものと期待される。

E. 結論

血管病モデルマウスの作製と登録も２７系統以上の成果を生み、バンクとしての機能（登録・分譲）機能も確立できた。

F. 健健康危険情報
なし

G. 研究発表
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H. 知的財産権の出願・登録状況

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 なし

別　紙　4

研究成果の刊行に関する一覧表

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Gab family proteins are essential for postnatal maintenance of cardiac function via neuregulin-1/ErbB signaling

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Grb2-associated binder (Gab) family of scaffolding adaptor proteins coordinate signaling cascades downstream of growth factor and cytokine receptors. In the heart, among EGF family members, neuregulin-1β (NRG-1β, a paracrine factor produced from endothelium) induced remarkable tyrosine phosphorylation of Gab1 and Gab2 via erythroblastic leukemia viral oncogene (ErbB) receptors. We examined the role of Gab family proteins in NRG-1β/ErbB-mediated signal in the heart by creating cardiomyocyte-specific Gab1/Gab2 double knockout mice (DKO mice). Although DKO mice were viable, they exhibited marked ventricular dilatation and reduced contractility with aging. DKO mice showed high mortality after birth because of heart failure. In addition, we noticed remarkable endocardial fibroelastosis and increase of abnormally dilated vessels in the ventricles of DKO mice. NRG-1β induced activation of both ERK and AKT in the hearts of control mice but not in those of DKO mice. Using DNA microarray analysis, we found that stimulation with NRG-1β upregulated expression of an endothelium-stabilizing factor, angiopoietin 1, in the hearts of control mice but not in those of DKO mice, which accounted for the pathological abnormalities in the DKO hearts. Taken together, our observations indicated that in the NRG-1β/ErbB signaling, Gab1 and Gab2 of the myocardium are essential for both maintenance of myocardial function and stabilization of cardiac capillary and endocardial endothelium in the postnatal heart.

Introduction

Dilated cardiomyopathy (DCM) is an common cause of heart failure. Epidemiological studies suggest that 25%–30% of DCM is inherited. Among the mutations associated with DCM in humans and mice, several involve genes encoding cytoskeletal proteins and sarcomere-related proteins (1); however, mutations in these known genes account for only a minor proportion of the heritable cardiomyopathies in humans. Cardiac function is maintained by cytokine- and growth factor–triggered intracellular signaling. Genetically modified mice, in which intracellular signaling molecules are either activated or perturbed, also exhibit cardiac dysfunction, suggesting that coordination of signal transduction systems is critical for the preservation of cardiac function (2).

The Grb2-associated binder (Gab) family proteins, which serve as scaffolding adaptor proteins, crucially intervene between receptors and intracellular signaling molecules to coordinate the signaling cascades of cytokines, growth factors, antigens, and numerous other molecules (3–5). Multiple phosphorylated tyrosine residues of Gab proteins become docking sites for Src homology-2 domain-containing molecules. Docking of Gab to tyrosine phosphatase SHP2 and the p85 regulatory subunit of PI3K leads to the activation of ERK and AKT, respectively (4, 5). Three Gab family members, Gab1, Gab2, and Gab3, have been identified in mammals and are structurally similar (4, 5). Conventional Gab1 knockout (Gab1KO) mice display embryonic lethality with impaired development of heart, placenta, skin, and muscle (6, 7). Gab2KO mice do not show any obvious developmental defects but display impaired allergic responses and osteoclast defects (8–11). Gab3KO mice exhibit no obvious phenotype (12).

We previously demonstrated the importance of Gab1-ERK5 signaling in cardiomyocyte hypertrophy through the leukemia inhibitory factor–gp130–dependent (LIF-gp130–dependent)

Nonstandard abbreviations used: Ang1, angiopoietin 1; ANP, atrial natriuretic peptide; DCM, dilated cardiomyopathy; DKO, cardiomyocyte-specific Gab1/Gab2 double knockout; EFE, endocardial fibroelastosis; EphA4, Eph receptor A4; ErbB, erythroblastic leukemia viral oncogene; Gab, Grb2-associated binder; Gab1CKO, cardiomyocyte-specific Gab1 conditional knockout; Gab1KO, conventional Gab1 knockout; HB-EGF, heparin-binding EGF-like growth factor; LIF, leukemia inhibitory factor; α-MHC, α-myosin heavy chain; NRG-1, neuregulin-1; α-SKA, skeletal α-actin; TSP1, thrombospondin 1.

Conflict of interest: The authors have declared that no conflict of interest exists.

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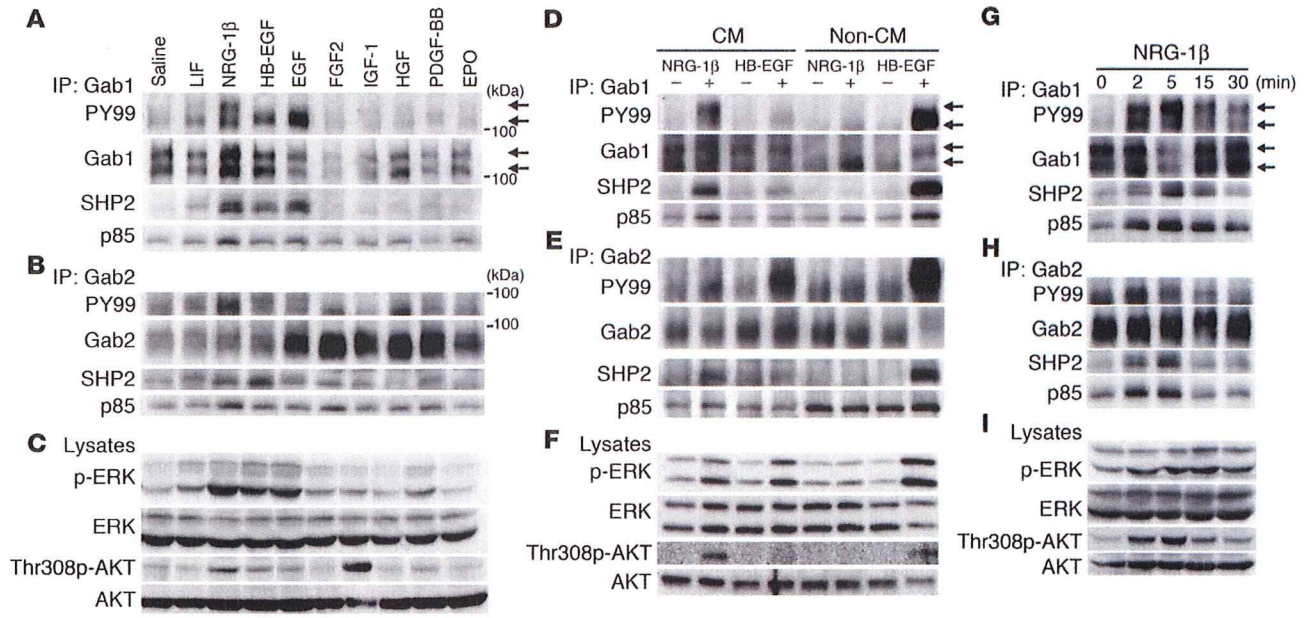

Figure 1

Gab1 and Gab2 are engaged in coordination of NRG-1β/ErbB signaling pathway in the myocardium. Tyrosine phosphorylation of Gab1 (**A**) and Gab2 (**B**) and their association with SHP2 and p85 were analyzed by IP of the heart lysates. Mouse heart lysates were prepared at 5 minutes after injection with the cytokines and growth factors listed at top. Heart lysates were subjected to IP with anti-Gab1 (**A**) or anti-Gab2 (**B**) serum, followed by IB analysis using the Ab indicated at the left. (**C**) Activation levels of ERK and AKT were assessed by phospho-specific Ab. Tyrosine phosphorylation of Gab1 (**D**) Gab2 (**E**) and their association with SHP2 and p85 was examined by IP of cell lysates from neonatal rat cardiomyocytes (CM) or noncardiomyocytes (non-CM) stimulated with either NRG-1β (50 ng/ml) or HB-EGF (50 ng/ml) for 5 minutes. IP complexes were subjected to IB using the Ab indicated at the left. (**F**) NRG-1β– and HB-EGF–dependent activation of ERK and AKT was examined in CM and non-CM as in **C**. Tyrosine phosphorylation of Gab1 (**G**) and Gab2 (**H**) and their association with SHP2 and p85 in the mouse hearts were analyzed after injection with 5 μg of NRG-1β as in **A** and **B**, respectively. Heart lysates were prepared at the indicated time after injection. Gab1 and Gab2 underwent tyrosine phosphorylation and associated with SHP2 and p85 in a time-dependent manner upon NRG-1β stimulation. (**I**) Activation of ERK and AKT were assessed as in **C**. Arrows denote 2 isoforms of Gab1. Representative blots of 3 experiments are shown. PY99, antibody recognizing phospho-tyrosine.

signaling pathway (13). Gab family proteins are also involved in EGF family–erythroblastic leukemia viral oncogene (EGF family–ErbB) receptor family signaling (6, 14, 15). EGF family–ErbB receptor signaling plays crucial roles in heart development and preservation of adult cardiac function (16, 17). Among the EGF family members, neuregulin-1 (NRG-1) (18) and heparin-binding EGF-like growth factor (HB-EGF) (19) are particularly important agonists for ErbB receptors on cardiomyocytes. NRG-1 serves as a paracrine factor that is shed from the endothelium and activates the ErbB4 homodimer or ErbB2/ErbB4 (also known as HER2/HER4) heterodimer on cardiomyocytes (16, 17, 20, 21). NRG-1–, ErbB2–, and ErbB4-deficient mice display embryonic lethality and similar defects in ventricular trabeculation (22–24). HB-EGF–deficient mice also display abnormal valvular development and cardiac dysfunction (25, 26).

The importance of ErbB signaling in the adult heart was first revealed by the unforeseen adverse effects of trastuzumab (Herceptin), a monoclonal Ab against ErbB2 used in the treatment of breast cancer. Trastuzumab induces heart failure when combined with anthracycline treatment (17, 27, 28). In addition to this clinical evidence, cardiomyocyte-specific ErbB2- and ErbB4-deficient mice both exhibit DCM (29–31). However, the precise intracellular signaling responsible for ErbB-regulated cardiac function is still unclear.

In the present study, we used myocardium-specific deletion of Gab family proteins in the mice to demonstrate that Gab1 and Gab2 in the myocardium are essential for transmitting the signal from NRG-1β/ErbB to directly maintain myocardial function and to subsequently stabilize capillary and endocardial endothelium in the postnatal heart.

Results

Gab1 and Gab2 are engaged in coordination of NRG-1β/ErbB signaling pathway in the myocardium. We aimed at exploring the function of Gab family proteins in the heart. Thus, we first examined the expression of Gab family transcripts by RT-PCR and detected the mRNA of Gab1 and Gab2, but not that of Gab3, in the murine heart (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI30651DS1). To elucidate how Gab1 and Gab2 are involved in the intracellular signaling in the heart, mice were injected with various cytokines and growth factors. Among these agonists, ErbB receptor-activating agonists, including NRG-1β, HB-EGF, and EGF, induced strong tyrosine phosphorylation of Gab1 and Gab2 and the subsequent association of Gab1 and Gab2 with SHP2 and p85 (Figure 1, A and B). We identified 2 Gab1 isoforms, high-molecular weight (high-MW) Gab1 (120–130 kDa) and low-MW Gab1 (100 kDa). Notably, the high-MW Gab1 underwent tyrosine phosphorylation

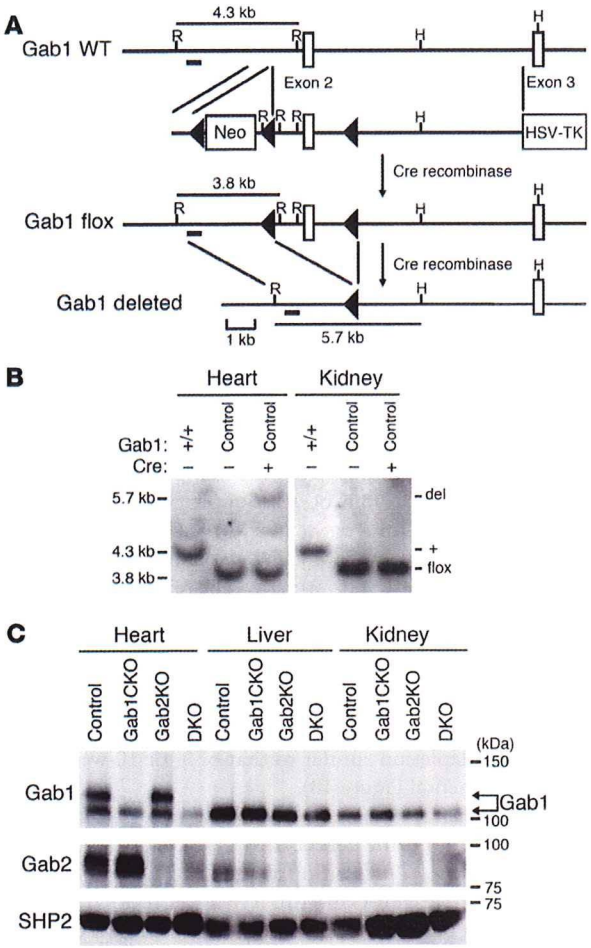

Figure 2
Generation of DKO mice. (**A**) Schematic illustration of genomic structure of the *Gab1* wild-type, *Gab1* flox, and *Gab1*-deleted alleles and a targeting vector. *lox*P sequences are indicated by black triangles. Restriction enzyme sites for *Eco*RI and *Hind*III are indicated as R and H, respectively. Fragments detected by the probe (short bold line) used for Southern blot analysis after digestion of genomic DNA with *Eco*RI and *Hind*III are indicated as solid lines measuring 4.3 kb, 3.8 kb, and 5.7 kb. HSV-TK, herpes simplex virus–thymidine kinase. (**B**) Southern blot analysis demonstrated recombination of the $Gab1^{flox}$ allele in the heart, but not in the kidney, of $Gab1^{flox/flox}$ mice, which possessed the α-*MHC–Cre* allele. (**C**) Following IP, expression of Gab1 and Gab2 was examined by IB using anti-Gab1 (top row) and anti-Gab2 (middle row) serums. SHP2 was examined as a loading control (bottom row). Note that 2 isoforms of Gab1 were detected at the different MW exclusively in the heart (arrows) and that the high-MW Gab1 isoform in the heart was completely depleted in Gab1CKO and DKO. The low-MW Gab1 was also reduced by 80% in the heart of Gab1CKO and DKO mice compared with control and Gab2KO mice.

upon stimulation exclusively with NRG-1β, while low-MW Gab1 was phosphorylated by NRG-1β, HB-EGF, and EGF (Figure 1A). We confirmed that the high-MW Gab1 is a cardiac-specific isoform using molecular mass spectrometric analysis, which showed that the high-MW band that was recognized by anti-Gab1 Ab in Western blot analysis indeed contained the partial amino acid sequence of Gab1 (Supplemental Figure 2, A–C). Activation of both ERK and AKT was found only when stimulated with NRG-1β, HB-EGF, and EGF (Figure 1C), although activation of AKT was most strongly induced by IGF-1.

We examined whether the difference in Gab1 phosphorylation was due to the diversity of the cell types. To distinguish the signaling processes in cardiomyocytes from those in noncardiomyocytes, including fibroblasts, endothelial cells, and vascular smooth muscle cells in the heart, we analyzed the action of NRG-1β and HB-EGF in neonatal rat cardiomyocytes and noncardiomyocytes that had been isolated using the Percoll gradient method (32). NRG-1β induced tyrosine phosphorylation of Gab1 and Gab2, the subsequent association of Gab1 and Gab2 with SHP2 and p85, and the activation of ERK and AKT in cardiomyocytes but not in noncardiomyocytes (Figure 1, D–F). In clear contrast, HB-EGF induced those changes more strongly in noncardiomyocytes than in cardiomyocytes (Figure 1, D–F). It should be noted that tyrosine phosphorylation of the high-MW Gab1 in cardiomyocytes was induced after stimulation with NRG-1β but not with HB-EGF (Figure 1D). These findings suggest that NRG-1β acts as a highly selective agonist for cardiomyocytes, in agreement with previous reports (33).

Therefore, we focused on the NRG-1β–dependent signaling pathway through Gab1 and Gab2 in the murine hearts. Gab1 and Gab2 underwent tyrosine phosphorylation and associated with SHP2 and p85 after injection with NRG-1β in a time-dependent manner (Figure 1, G and H). In addition, both ERK and AKT were also activated by NRG-1β in a time-dependent manner (Figure 1I). We also checked the activation of ErbB family receptors of murine hearts stimulated with NRG-1β. NRG-1β induced tyrosine-phosphorylation of ErbB2 and ErbB4 but not that of ErbB1 (EGFR) or ErbB3 in accordance with a previous report in which cardiomyocytes were used in vitro (Supplemental Figure 3, A–D) (21). Furthermore, Gab1 associated with ErbB4 in a phosphorylation-dependent manner after injection with NRG-1β (Supplemental Figure 3E). These data suggest the engagement of Gab family proteins in the coordination of NRG-1β/ErbB signaling pathway.

Generation of cardiomyocyte-specific Gab1 conditional knockout mice. To elucidate the function of Gab family proteins in myocardium, we first generated cardiomyocyte-specific *Gab1* conditional knockout (Gab1CKO) mice using the Cre-*loxP* system. Using homologous recombination in embryonic stem cells, we created a $Gab1^{flox}$ allele by introducing 2 loxP sites into introns flanking exon 2, which encodes part of the pleckstrin homology domain

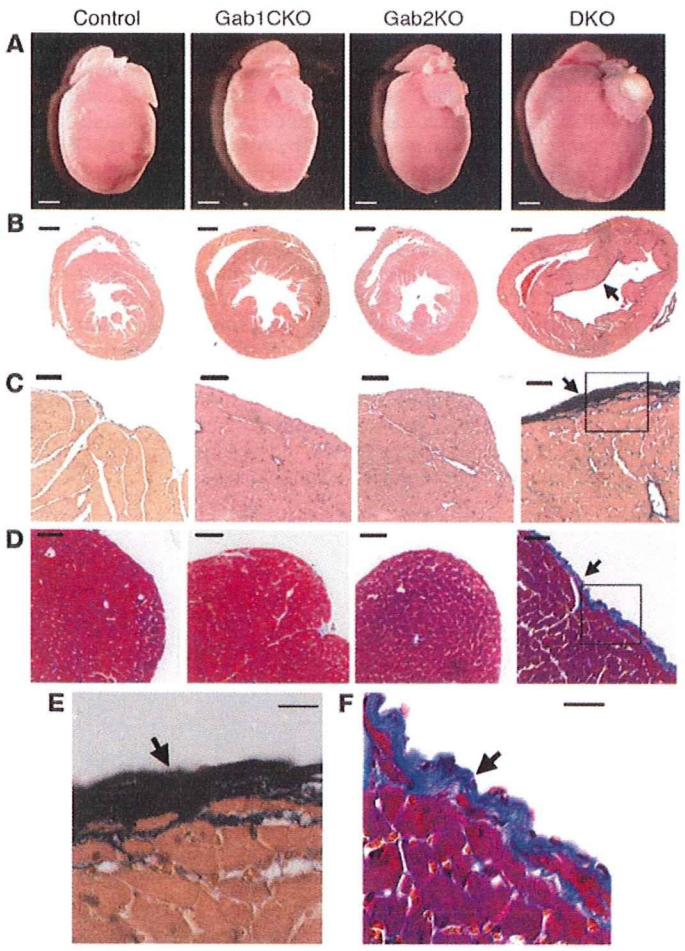

Figure 3
DKO mice display dilated cardiomyopathic features accompanied by EFE. (**A**) Representative images of whole hearts from 4 groups at 10 weeks of age. (**B**) Transverse sections of the hearts were stained using the elastica van Gieson method. DKO hearts showed marked biventricular dilation and slight wall thinning compared with the other 3 groups of hearts. (**C** and **E**) Higher magnification of elastica van Gieson–stained section of DKO heart shows the focal accumulation of elastic fibers (black) in the endocardium (arrows in **B** and **C**). (**D**) Masson's trichrome–stained section of DKO heart shows focal accumulation of collagen (blue) in the endocardium (arrow in **D**). (**E** and **F**) Boxed regions of **C** and **D**, respectively, are enlarged. Scale bars: 1 mm (**A** and **B**); 20 μm (**C**–**F**).

(Figure 2A). The protein expression of Gab1 in all tissues of mice homozygous for the Gab1-*loxP*–targeted allele (*Gab1*$^{flox/flox}$ mice) was almost the same level as in wild-type mice (data not shown). To cause recombination of the floxed allele exclusively in cardiomyocyte lineage, *Gab1*$^{flox/flox}$ mice were crossed with transgenic mice expressing α-*myosin heavy chain* promoter–driven Cre recombinase (α-*MHC–Cre* mice) (34, 35) (Figure 2A). We confirmed the Cre-mediated recombination during embryogenesis (E10.5 and E14.5) by crossing α-*MHC–Cre* mice with enhanced GFP reporter mice (Supplemental Figure 4A). The Gab1CKO (*Gab1*$^{flox/flox}$ α-*MHC-Cre(+)*) mice were born normally at the expected Mendelian frequency, whereas Gab1KO mice were embryonically lethal (6). In addition, the Gab1CKO mice displayed normal appearance and normal cardiac morphology at birth (Supplemental Figure 5A).

We observed the expected genetic recombination at the *Gab1* locus in the ventricles of Gab1CKO mouse hearts but not in other tissues (Figure 2B). In order to estimate the expression of Gab1 protein, immunoblot analyses were performed using the extracts from heart, liver, and kidney (Figure 2C). As described above, 2 isoforms of Gab1 proteins were detected in hearts, while low-MW Gab1 was commonly detected, suggesting that the high-MW Gab1 is a cardiac-specific isoform. Moreover, high-MW Gab1 protein was deleted in Gab1CKO hearts, suggesting that high-MW Gab1 is a product of the same *Gab1* gene that has low MW. In addition, we used Percoll gradient centrifugation to analyze the expression of Gab1 in cardiomyocytes and noncardiomyocytes isolated from neonatal rat hearts (32) and detected the high-MW isoform of Gab1 exclusively in cardiomyocytes (Supplemental Figure 2D).

In Gab1CKO mice, the high-MW Gab1 was completely deleted and the low-MW Gab1 was reduced to about 20% of control (*Gab1*$^{flox/flox}$) littermates. The residual low-MW Gab1 protein might be attributed to the noncardiomyocytes present in the heart. These data indicated the successful depletion of Gab1 in the cardiomyocytes (Figure 2C), because α-*MHC* promoter functions exclusively in the myocardium. In 3-day-old Gab1CKO mouse hearts, we detected an extent of Gab1 protein depletion similar to that of 3- or 10-week-old mice (Supplemental Figure 4B).

Generation of cardiomyocyte-specific Gab1/Gab2 double knockout mice. In murine hearts, mRNAs of Gab1 and Gab2 were detected by RT-PCR (Supplemental Figure 1). Gab2 can rescue the loss of Gab1 for activation of ERK in the EGF signaling pathway (36). We thus assumed that Gab2 might compensate for the deletion of Gab1 in the cardiomyocytes of Gab1CKO mice.

To completely deplete Gab family proteins in cardiomyocytes, Gab1CKO mice were crossed with Gab2KO mice. We created *Gab1*$^{flox/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(+)* mice by crossing *Gab1*$^{+/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(+)* mice with *Gab1*$^{flox/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(–)* mice in the final breeding. The offspring of these crossings were recovered at expected Mendelian ratios as follows: *Gab1*$^{+/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(–)* (n = 44; 24.6%); *Gab1*$^{+/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(+)* (n = 46; 25.7%); *Gab1*$^{flox/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(–)* (n = 39; 21.8%); *Gab1*$^{flox/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(+)* (n = 50; 27.9%). Thereafter, we analyzed the following 4 groups of mice: *Gab1*$^{flox/flox}$*Gab2*$^{+/+}$α-*MHC–Cre(–)* (control); *Gab1*$^{flox/flox}$*Gab2*$^{+/+}$α-*MHC–Cre(+)* (Gab1CKO); *Gab1*$^{flox/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(–)* (Gab2KO); and *Gab1*$^{flox/flox}$*Gab2*$^{-/-}$α-*MHC–Cre(+)* (DKO). Both Gab2KO and DKO mice displayed normal appearance and normal cardiac morphology at birth (Supplemental Figure 5A). Gab2 protein was completely depleted in the Gab2KO and DKO mice, indicating the successful depletion of Gab1 and Gab2 in the cardiomyocytes of DKO mice (Figure 2C).

DKO mice display dilated cardiomyopathic features accompanied by endocardial fibroelastosis. We performed gross morphological examination of the hearts of the 4 groups at 10 weeks of age because we did not find any morphological abnormalities in the hearts of Gab1CKO, Gab2KO, or DKO mice at birth (Supplemental Figure 5A). Although there was no morphological difference among Gab1CKO, Gab2KO, and control mice (Figure 3A), DKO mice exhibited significantly higher heart weight–to–body weight ratios

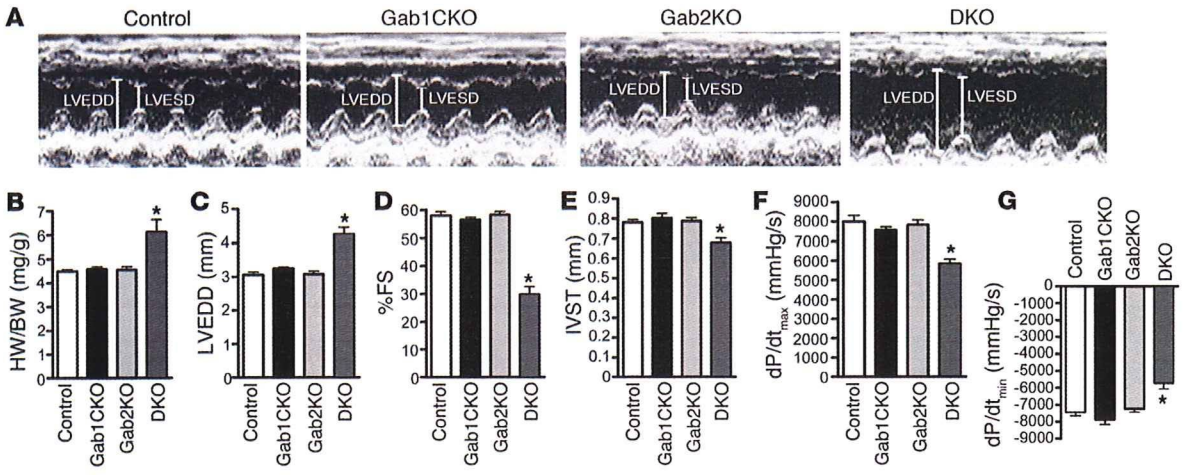

Figure 4

DKO mice exhibit dilated cardiomyopathic features. (**A**) Representative examples of M-mode echocardiographic images of LV from each group of mice at 10 weeks of age. LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension. (**B**) Heart weight/body weight (HW/BW) ratio of control mice (n = 9), Gab1CKO mice (n = 6), Gab2KO mice (n = 6), and DKO mice (n = 10) at 10 weeks of age. (**C**) LVEDD, (**D**) fractional shortening (%FS), and (**E**) interventricular septal thickness (IVST) of control mice (n = 8), Gab1CKO mice (n = 8), Gab2KO mice (n = 7), and DKO mice (n = 14) at 10 weeks of age. There were no significant differences in BW or heart rate among the 4 groups. (**F**) The maximum first derivative of LV pressure (LV dP/dt$_{max}$) and (**G**) the minimum first derivative of LV pressure (LV dP/dt$_{min}$) were obtained by catheterization of LV from right carotid artery in control mice (n = 7), Gab1CKO mice (n = 6), Gab2KO mice (n = 7), and DKO mice (n = 7) at 12 weeks of age. *P < 0.01 compared with all other genotypes.

than the other 3 groups without significant differences in body weight (Figure 3A and Figure 4B). Histological examination also demonstrated both left and right ventricular enlargement in DKO mice similar to DCM (Figure 3B).

A significant accumulation of elastic fibers and collagen was observed exclusively in the endocardium of DKO mice (Figure 3, B–F), while fibrotic replacement was not found in the interstitial spaces of the ventricles of DKO mice (Supplemental Figure 6, A and B). There was no significant increase in the number of apoptotic myocardial cells in the hearts of DKO mice compared with those of control mice (Supplemental Figure 7, A and B). The endocardial deposition of elastic fibers and collagen was not found in the neonates of DKO, but was found to some extent in all of the DKO mice after 3 weeks (Supplemental Figure 5A and data not shown). These endocardium-specific changes were coincident with the pathological features of endocardial fibroelastosis (EFE), the genetic causality of which has not been fully elucidated to date (37, 38). We further examined the vasculature in the heart by immunostaining with anti-vWF Ab. Intriguingly, we found abnormally dilated vessels positively stained with anti-vWF Ab exclusively in the LV of DKO mice but not in those of control, Gab1CKO, or Gab2KO mice (Figure 5A). These dilated vessels in DKO mice exhibited the impairment in recruitment of α-SMA–positive VSMCs (Figure 5, B and C). These findings indicate that the maintenance system for both endocardial and vascular endothelium might be disturbed in the DKO mouse hearts. Furthermore, EFE and increased abnormal vessels in the hearts of DKO mice were indirectly ascribed to the lack of Gab1 and Gab2 in the myocardium because there was no abnormality in the other 3 groups.

We assessed in vivo cardiac function by echocardiography and cardiac catheterization. Echocardiography revealed a significant increase in LV end-diastolic dimension (Figure 4, A and C), decreased fractional shortening (Figure 4, A and D), and decreased interventricular septal wall thickness (Figure 4E) in 10-week-old DKO mice compared with age-matched mice of the other 3 groups. Although we did not find a significant changes of LV end-diastolic dimension or fractional shortening between the DKO and control mice at 3 weeks of age, we did observe these changes after 6 weeks of age (Supplemental Figure 8, A and B). Consistent with the echocardiographic findings, cardiac catheterization at 12 weeks of age revealed a marked reduction of the maximum first derivative of LV pressure exclusively in DKO (Figure 4F), demonstrating a reduction in myocardial contractility of the DKO hearts. The accompanying reduction of the minimum first derivative of LV pressure in the DKO mouse hearts indicated the impairment of LV relaxation (Figure 4G). There were no significant differences in heart rate or LV peak pressure among the 4 groups (data not shown). This relaxation failure was supported by the electron microscopic findings. We noticed that sarcomere length was reduced in the DKO mouse hearts, which indicated the hypercontraction phenotype (39), although we could detect slight changes in the mitochondria of DKO mouse hearts (Supplemental Figure 7, C and D). In agreement with the reduced contractility and relaxation reflecting heart failure, the fetal cardiac gene program was reactivated, as evidenced by the significant increase in both *atrial natriuretic peptide* (*ANP*) and *skeletal α-actin* (*α-SKA*) mRNAs in DKO mice (Figure 6, A–C).

Approximately 70% of the DKO mice died, presumably of heart failure accompanied by pleural effusion, between 3 and 72 weeks of age (Figure 6D). We observed remarkably dilated ventricles in DKO mice that had died of heart failure (Supplemental Figure 5B, right panel). The other 3 groups of mice lived normally during the observation period of 500 days (Figure 6D). In agreement with this survival analysis, we did not observe any enlargement of the hearts of Gab1CKO and Gab2KO mice at 300 and 500 days of age. (Supplemental Figure 5B and data not shown). These data indicate that depletion of both Gab1 and Gab2 in the myocardium result in DCM-like phenotype accompanied by EFE.

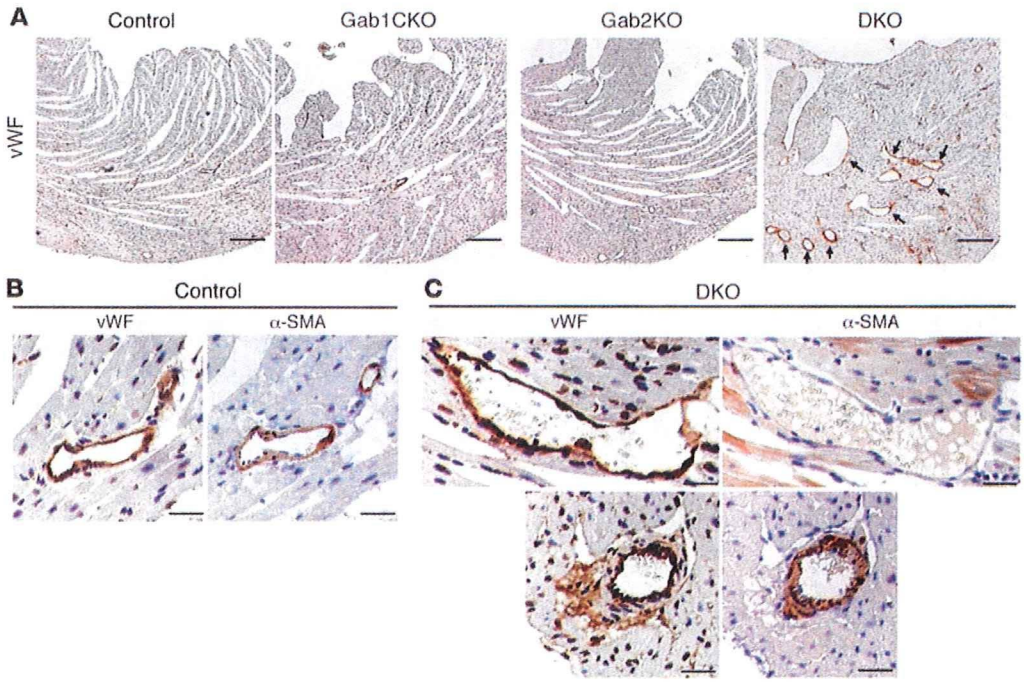

Figure 5

DKO mice display vascular abnormalities in the ventricles. (**A**) Heart sections from 4 groups of mice at 6 weeks of age were immunostained with anti-vWF Ab. vWF-positive, abnormally dilated vessels were observed in the left ventricles of DKO mice (arrows) but not in those of control, Gab1CKO, or Gab2KO mice. Representative photographs are shown. (**B** and **C**) Heart sections from control (**B**) and DKO (**C**) mice at 6 weeks of age were immunostained with anti-vWF and anti–α-SMA Abs. The abnormally dilated vessels in DKO mice were not surrounded by α-SMA–positive VSMCs in most cases (**C**, top panels), although vessels of normal diameter near the epicardium in DKO mice were surrounded by α-SMA–positive VSMCs (**C**, bottom panels) as observed in control mice (**B**). Representative images are shown. Scale bars: 200 μm (**A**); 20 μm (**B** and **C**).

Gab1 and Gab2 are required for NRG-1β/ErbB signaling in the heart. To determine requirements of Gab1 and Gab2 in NRG-1β–triggered signaling in the myocardium, we examined the activation of ERK and AKT after injection of NRG-1β. NRG-1β–induced activation of ERK and AKT was completely abrogated in DKO mice but not in the other 3 groups (Figure 7, A–C), suggesting a compensatory function of Gab1 and Gab2 in the heart. Consistently, tyrosine phosphorylation of Gab1 and subsequent association with SHP2 and p85 were observed in control and Gab2KO mice but not in Gab1CKO or DKO mice (Figure 7D). Tyrosine phosphorylation of Gab2 and subsequent association with SHP2 and p85 were conversely observed in control or Gab1CKO mice but not in Gab2KO or DKO mice (Figure 7E). Tyrosine phosphorylation of ErbB2 and ErbB4 was comparable among the 4 groups (Figure 7F). IGF-1– and HB-EGF–dependent activation of ERK and AKT were not affected in the hearts of DKO mice (Supplemental Figure 9, A and B). These data indicate that Gab1 and Gab2 are required exclusively for NRG-1β/ErbB signal-dependent activation of ERK and AKT in the heart.

Angiopoietin 1 upregulation induced by NRG-1β is impaired in Gab1/Gab2-deficient myocardium. Because we observed no cardiac abnormalities in Gab2KO mice, we determined that the primary cause of EFE and abnormal vessels in DKO mouse hearts was not the lack of Gab2 in endothelial cells. To identify the potential signal defect that caused EFE and malformed vessels downstream of the NRG-1β/ErbB–Gab1/Gab2 signaling pathway in the myocardium, we used microarrays to carry out a global survey of mRNA in control and DKO mice treated with or without NRG-1β for 8 hours. We found several transcripts that were upregulated by stimulation with NRG-1β in the hearts of control mice but not in those of DKO mice (Figure 8A). Among these transcripts presented in the cluster diagram, we considered thrombospondin 1 (TSP1) and angiopoietin 1 (Ang1) to be potential paracrine factors from myocardium and Eph receptor A4 (EphA4) to be important for the intercellular communication between cardiomyocytes and surrounding cells.

To address the pathogenesis of endocardial and vascular abnormalities observed in DKO mouse hearts, we focused on Ang1 because it has an important role in maturation of both vascular endothelium and endocardial endothelium in vivo (40–42). We confirmed by northern blot analysis that NRG-1β upregulated *Ang1* mRNA in the hearts of control mice, but not DKO mice (Figure 8, B and C). NRG-1β consistently induced significant upregulation of *Ang1* mRNA in cultured cardiomyocytes but not in noncardiomyocytes (Figure 8, D–F). In association with defective expression of Ang1, CD31-positive capillary density was significantly decreased in the LV of DKO mice compared with control (Figure 8, G and H). Taken together, these findings suggest that the lack of NRG-1β–induced upregulation of Ang1 might be one of the possible causes for pathogenesis of EFE and abnormal vasculatures in DKO mouse hearts.

Discussion

To our knowledge, the present study is the first to reveal the essential roles of Gab family proteins for NRG-1β/ErbB signaling pathway in the heart. Gab1 and Gab2 were markedly tyro-

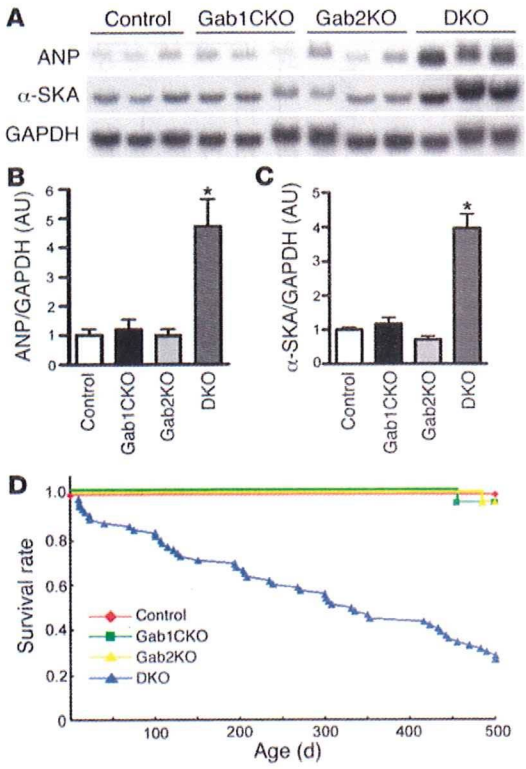

Figure 6
DKO mice die of heart failure. (**A**) Northern blot analyses of the hearts from control, Gab1CKO, Gab2KO, and DKO mice (n = 3 for each group) at 12–14 weeks of age showed the increased expression of mRNAs for *ANP* and *α-SKA* in DKO mice. *GAPDH* mRNA was also measured for sample loading control. (**B** and **C**) The relative levels of *ANP* and *α-SKA* mRNA (normalized to *GAPDH* mRNA levels) were quantified from 3 mouse hearts in each group. (*P < 0.01 compared with all other groups.) (**D**) Kaplan-Meier curves showing survival rate in control mice (n = 30), Gab1CKO mice (n = 30), Gab2KO mice (n = 30), and DKO mice (n = 66) mice by 500 days. The number of dead DKO mice was 48 (72.7%); P < 0.001 for DKO versus control, Gab1CKO, and Gab2KO mice by log-rank test.

sine phosphorylated in the myocardium after stimulation with NRG-1β among various growth factors and cytokines. Tyrosine-phosphorylated Gab1 and Gab2 subsequently associated with SHP2 and p85, resulting in strong activation of both ERK and AKT in the myocardium. NRG-1β–dependent activation of ERK and AKT was almost completely abrogated in the DKO mouse hearts. In agreement with NRG-1β–dependent downstream signaling defects, DKO mice displayed DCM-like phenotypes and EFE with aging. Interestingly, DKO mouse hearts also displayed abnormally dilated vessels with the loss of VSMCs. To address the mechanism for the abnormality in endocardial/vascular endothelium in DKO mouse hearts, we performed DNA microarray analysis and found several vasculature-regulating gene transcripts, such as Ang1, upregulated by NRG-1β in control, but not in DKO, mouse hearts. Thus, Gab family proteins mediate NRG-1β–dependent stabilization of endocardial/vascular endothelium through the paracrine system from cardiomyocytes in the heart.

Gab1 and Gab2 are specifically required for coordination of NRG-1β/ErbB–dependent signaling pathway in the myocardium. NRG-1β shed from endothelial cells activates ErbB2/ErbB4 heterodimer or ErbB4 homodimer on the cardiomyocytes (16, 17, 21). Consistent with this notion, we found that NRG-1β induced prominent tyrosine phosphorylation of Gab1 and Gab2 in cardiomyocytes but not in noncardiomyocytes. In addition, the cardiomyocyte-specific, high-MW isoform of Gab1 was tyrosine phosphorylated after stimulation with NRG-1β but not with other agonists including HB-EGF and EGF. It has been reported that HB-EGF–deficient mice develop heart failure (25, 26). Given that HB-EGF induced a much stronger tyrosine phosphorylation of Gab1 and Gab2 in noncardiomyocytes than in cardiomyocytes in our study and that valvular structures are developed from noncardiomyocytes (19), the heart failure observed in HB-EGF–deficient mice might have resulted from abnormal signaling in the development of the valvular apparatus. Therefore, the cardiac phenotypes observed in DKO mice were mainly ascribable to the defects of the NRG-1β/ErbB signaling pathway in the myocardium. Consistent with this, similar DCM-like phenotypes are found in cardiac-specific ErbB2- and ErbB4-deficient mice (29–31).

NRG-1β activates both ERK and PI3K/AKT pathways in cardiomyocytes in vitro, both of which have been implicated in modulation of cell survival and protein synthesis (21, 43). NRG-1β actually induced strong activation of ERK and AKT in the hearts of control, but not DKO, mice. This finding provides what we believe to be the first in vivo evidence that Gab1 and Gab2 are required for transmission of the NRG-1β/ErbB signal to downstream signaling pathways, ERK and AKT. DKO mice progressively developed DCM phenotypes, demonstrating clearly that Gab1 and Gab2 were essential for maintenance of myocardial function through transmission of NRG-1β/ErbB signaling pathway (Figure 9).

DKO mice also exhibited abnormal deposition of elastic fibers and collagen specifically in the endocardium, reminiscent of the pathological features observed in primary EFE. Clinically, primary EFE is found mainly in infants, children, and adolescents and is frequently accompanied by contractile deterioration similar to DCM. Although there have been some reports suggesting the heritable causality of primary EFE (37, 38), the precise pathogenetic mechanisms have not been elucidated to date. These DKO mice may provide the first mouse model of EFE. Further genetic analysis of cardiac-specific isoform of Gab1 will certainly contribute to our understanding of the pathogenesis of EFE.

DKO mouse hearts also displayed abnormal vasculatures as well as EFE. Microarray analysis enabled us to identify several transcripts that were upregulated by NRG-1β in the control hearts but not in DKO hearts. Among these transcripts selected in the cluster analysis, TSP1, EphA4, and Ang1 have been reported to be involved in the intercellular-dependent vascular regulation (40, 44, 45). Intriguingly, NRG-1β/ErbB2/ErbB4 signaling, Ang1/Tie2 signaling, VEGF/VEGFR2 signaling, and serotonin-mediated (5-HT_{2B}–mediated) signaling are required for the proper maturation of endocardium (16, 17, 40, 46, 47). Moreover, Ang1- or Tie2-deficient mice exhibit embryonic lethality accompanied by abnormally dilated vessels as well as defects in the endocardium (40, 42, 48). Furthermore, we demonstrated for the first time that postnatal cardiomyocytes are important Ang1-producing cells, whereas Ang1 has been believed to be mainly secreted from vascular mural cells such as pericytes and VSMCs (40, 41). Thus, we could pro-

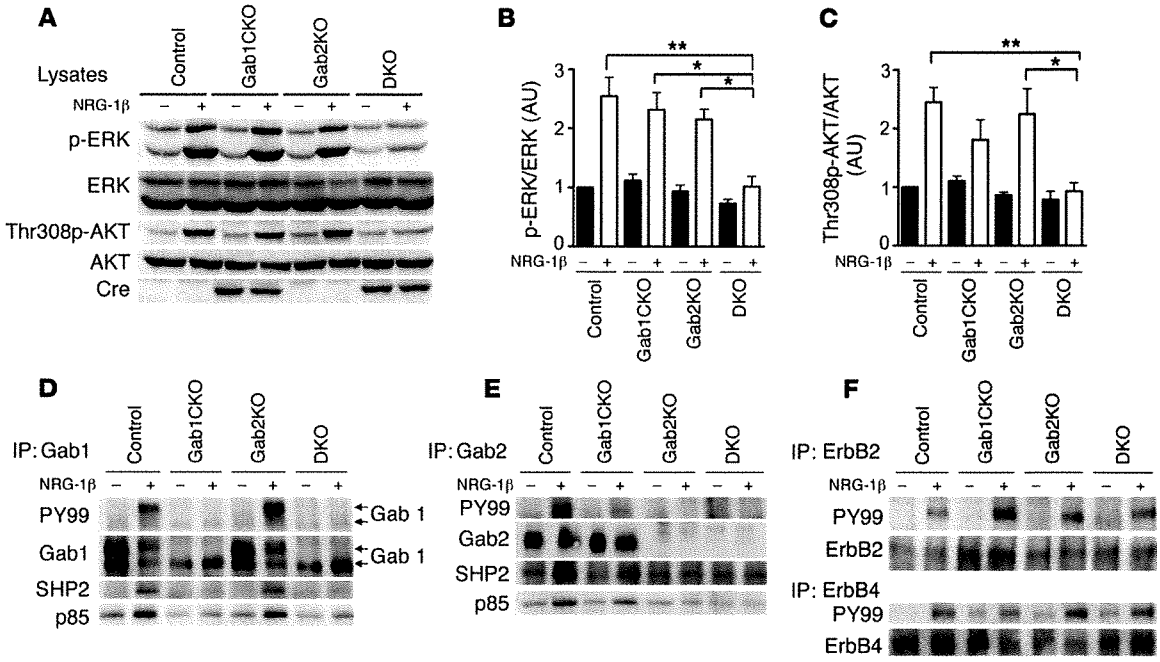

Figure 7

Gab1 and Gab2 are required for NRG-1β–dependent ERK and AKT activation in the heart. (**A**) NRG-1β–induced activation of ERK and AKT in the hearts from the indicated mice was assessed using phospho-specific Abs. Activation of ERK and AKT was exclusively attenuated in DKO hearts compared with the other 3 groups. Representative blots of 4 experiments are shown. (**B**) Phosphorylation of ERK was quantified against total ERK ($n = 4$). (**C**) Phosphorylation of AKT was quantified against total AKT ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$ for the indicated groups. Tyrosine phosphorylation of Gab1 (**D**) and Gab2 (**E**) and their association with SHP2 and p85 in hearts from the 4 groups of mice after injection with NRG-1β was examined as in Figure 1, A and B. Arrows in **D** denote the 2 isoforms of Gab1. (**F**) Tyrosine phosphorylation of ErbB2 (upper panels) and ErbB4 (lower panels) in hearts from the 4 groups were assessed at 5 minutes after NRG-1β injection. Tyrosine phosphorylation of ErbB receptors in the murine hearts upon NRG-1β stimulation was examined by IP with anti-ErbB2 or anti-ErbB4 Ab, followed by IB with the Abs indicated at the left.

pose that the defective expression of Ang1 might be involved in the pathogenesis of EFE and abnormal vessels in DKO hearts, though we cannot exclude the possibility that other vasculature-regulating genes, such as TSP1 and EphA4, play important roles in endocardial maintenance. Cardiac-specific gene ablation of Ang1 would be helpful to understand its importance in cardiomyocyte-endothelial cell interactions.

So far, it has been well established that NRG-1 functions as a cytoprotective growth factor in cardiomyocytes (17, 21, 43). Here, our findings propose a novel function of NRG-1; NRG-1 regulates vascular homeostasis through the paracrine expression of endothelium stabilization factors, such as Ang1, via Gab family proteins. Importantly, accumulating evidence has revealed that normal endothelial function is required for the maintenance of myocardial function (16). Collectively, Gab1 and Gab2 in the myocardium are essential for both maintenance of myocardial function and stabilization of capillary or endocardial endothelium through transmission of NRG-1β/ErbB signaling (Figure 9).

Methods

Materials. Anti-phospho-p44/p42 ERK (Thr202/Tyr204), anti-phospho-AKT (Thr308), and anti-AKT Abs were purchased from Cell Signaling Technology. The use of anti-Gab1 and anti-Gab2 serums in IP was described previously (13, 49). The Abs against Gab1, Gab2, and p85 used in IB analysis were from Millipore; Abs against antibody recognizing phospho-tyrosine (PY99), ERK1, ERK2, and SHP2 were from Santa Cruz Biotechnology Inc.; Abs against vWF and α-SMA were from Dako; the Ab against CD31 was from BD Biosciences — Pharmingen; and the Ab against Cre was from EMD Biosciences. Collagenase, Percoll, recombinant NRG-1β (NRG-1β EGF domain; sold as heregulin-β1), HGF, and PDGF-BB were from Sigma-Aldrich. HB-EGF and EGF were from R&D Systems. FGF2 was from EMD Biosciences. LIF was from Millipore. IGF-1 and erythropoietin were kindly provided by Astellas Pharma and Chugai Pharmaceutical Co., respectively.

Cell cultures. Primary cultures of neonatal rat cardiomyocytes were prepared from ventricles of 1- to 2-day-old Wistar rats (Kiwa Jikken Dobutsu) on Percoll gradient as described previously (32). Briefly, ventricles were isolated from neonatal rats and treated with trypsin and collagenase for 30 minutes at 37°C. Isolated cells were suspended in 58.5% Percoll in HBSS (20 mM HEPES, 116 mM NaCl, 12.5 mM NaH_2PO_4, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$; pH 7.35) and added to the discontinuous gradient consisting of 40.5% and 58.5% Percoll in HBSS. After centrifugation at 1,400 *g* for 30 minutes at 15°C, the cardiomyocytes were collected from the interface of the discontinuous Percoll gradient and further enriched by preplating for 60 minutes on noncoated dishes. Unattached cells were cultured as cardiomyocytes in M-199 (Invitrogen) with 10% FBS. Attached cells were cultured as noncardiomyocytes in DMEM with 10% FBS. Immunocytochemical examination with anti-sarcomeric α-actinin Ab (Sigma-Aldrich) revealed that more than 95% cultured cells in the cardiomyocyte fraction were sarcomeric α-actinin–positive cardiomyocytes (data not shown). The population of noncardiomyocytes is described in the supplemental information.