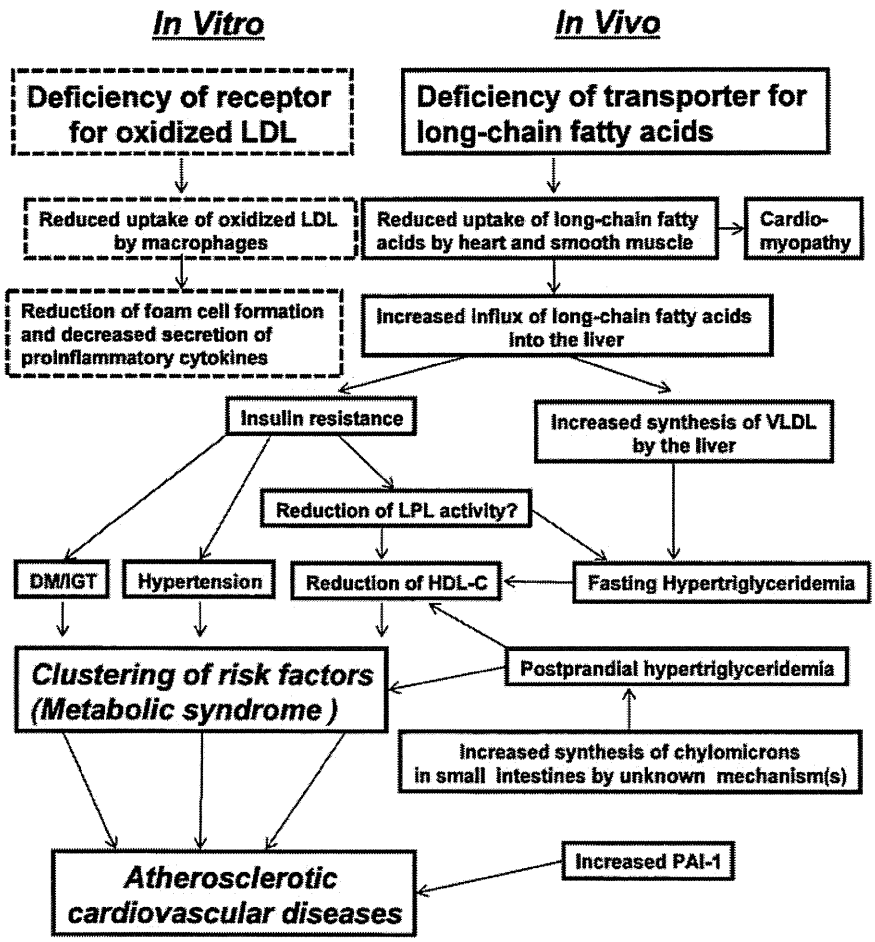

Fig. 5. Possible molecular mechanisms for enhanced atherosclerosis in human CD36-D

In vitro, CD36 deficiency causes the reduced uptake of oxidized LDL by macrophages, leading to decreased foam cell formation and secretion of proinflammatory cytokines; however, *in vivo*, CD36 deficiency results in reduced uptake of long-chain fatty acids (LCFA) by the heart and skeletal muscles, causing impaired metabolism of LCFA by the liver and circulation. These abnormalities as a whole may eventually lead to atherosclerotic cardiovascular diseases.

be linked with an increased risk of atherosclerotic cardiovascular diseases in obesity, insulin resistance and diabetes mellitus[46]. The increase of PAI-I was partly attributed to the accumulation of abdominal visceral fat[47]. Yanai *et al.*[48] reported elevated PAI-I levels in patients with CD36-D, although the mechanism was speculated to be linked to abnormal fatty acid metabolism.

Taken together, as illustrated in **Fig. 5**, despite the anti-atherosclerotic aspects of monocyte-derived macrophages from CD36-D patients due to the reduced uptake of oxidized LDL and decreased secretion of proinflammatory cytokines *in vitro*, the pro-atherogenic profiles *in vivo* may exceed the anti-atherosclerotic properties, thus enhancing the development of atherosclerosis. These pro-atherogenic profiles of CD36-D patients include: 1) increased lipoprotein remnants and postprandial hyperlipidemia, 2) reduced serum HDL-C levels, 3) increased FFA levels because of deficiency of LFCA transporter, 4) insulin resistance and impaired glucose metabolism, 5) hypertension, and 6) increased levels of PAI-I. These risk parameters may cluster and interact, finally leading to the marked enhancement of atherosclerosis; therefore, early screening and detection of CD36-D patients and assessment of atherosclerotic cardiovascular diseases are essential, especially in a population such as the Japanese in which their frequency is extremely high. Fur-

ther investigations into the molecular and vascular biological mechanisms of the progression of atherosclerosis in patients with CD36-D may be necessary in future studies.

Conclusions

Patients with CD36-D are associated with severe and enhanced atherosclerotic diseases. The morbidity of CAD is significantly higher in patients with CD36-D than in healthy subjects, and the frequency of CD36-D is significantly higher in patients with CAD than in healthy subjects. The clustering of atherogenic metabolic profiles such as dyslipidemia, including the accumulation of FFA and remnants, hypertension and insulin resistance, may enhance atherogenicity in patients with CD36-D.

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Conflicts of Interest

S. Yamashita has received consultancy fees from Otsuka Pharmaceutical Company and Skylight Biotech Co. The other co-authors have nothing to disclose.

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IMAGES IN CARDIOLOGY

Cardiac Allograft Vasculopathy Progression Associated With Intraplaque Neovascularization

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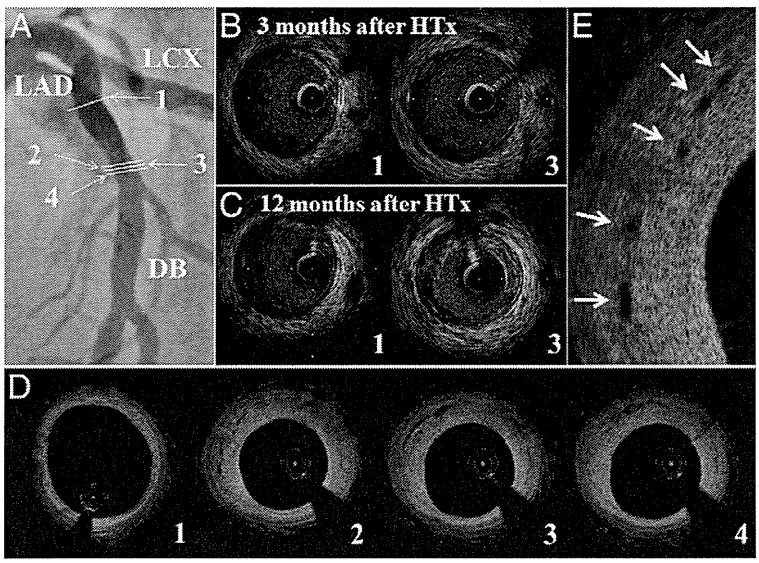

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A 52-year-old man underwent scheduled cardiac catheterization for assessment of cardiac allograft vasculopathy (CAV) at 3 and 12 months after heart transplantation (HTx). Intravascular ultrasound showed only mild CAV in the left anterior descending coronary artery (LAD) at 3 months (**B**, Online Video 1). However, at 12 months, CAV progression was detected, predominantly in the proximal LAD (**A and C3**, Online Video 2). Corresponding optical coherence tomography images revealed the presence of many no-signal tubuloluminal structures within the fibrous plaque in serial frames. Parts of those structures were found to be connected to the vessel lumen, indicating neovascularization (**D2, D3, D4, and E [arrows]**, Online Video 3). No neovascularization was observed in lesions without CAV progression **(C1 and D1)**. To our best knowledge, this is the first reported case demonstrating CAV progression associated with intraplaque neovascularization in vivo. Intraplaque neovascularization could play an important role in CAV progression. DB = diagonal branch; LCX = left circumflex coronary artery.

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Review Article

Gab Docking Proteins in Cardiovascular Disease, Cancer, and Inflammation

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The docking proteins of the Grb2-associated binder (Gab) family have emerged as crucial signaling compartments in metazoans. In mammals, the Gab proteins, consisting of Gab1, Gab2, and Gab3, are involved in the amplification and integration of signal transduction evoked by a variety of extracellular stimuli, including growth factors, cytokines, antigens, and other molecules. Gab proteins lack the enzymatic activity themselves; however, when phosphorylated on tyrosine residues, they provide binding sites for multiple Src homology-2 (SH2) domain-containing proteins, such as SH2-containing protein tyrosine phosphatase 2 (SHP2), phosphatidylinositol 3-kinase regulatory subunit p85, phospholipase Cγ, Crk, and GC-GAP. Through these interactions, the Gab proteins transduce signals from activated receptors into pathways with distinct biological functions, thereby contributing to signal diversification. They are known to play crucial roles in numerous physiological processes through their associations with SHP2 and p85. In addition, abnormal Gab protein signaling has been linked to human diseases including cancer, cardiovascular disease, and inflammatory disorders. In this paper, we provide an overview of the structure, effector functions, and regulation of the Gab docking proteins, with a special focus on their associations with cardiovascular disease, cancer, and inflammation.

1. Introduction

The mammalian Grb2-associated binder (Gab) proteins are homologs of *Drosophila* DOS (Daughter Of Sevenless) and *Caenorhabditis elegans* SOC-1 (Suppressor Of Clear). These proteins define a family of docking proteins closely related to the insulin receptor substrate (IRS-1, IRS-2, IRS-3), fibroblast growth factor substrate (FRS2), linker of T cell (LAT), and downstream of kinase (Dok) families [1]. In contrast to adaptor proteins such as growth factor receptor bound protein 2 (Grb2) and Shc, which are usually smaller and often function as a molecular bridge between two proteins in the assembly of larger protein complexes, docking proteins contain a membrane-targeting region at the N-terminus, binding sites for src homology 3 (SH3) domain-containing proteins, and multiple tyrosine phosphorylation sites that, when phosphorylated, function as binding sites for the src homology 2 (SH2) domains of a variety of effectors. Consequently, the docking proteins are significantly larger than adaptor proteins. In addition, docking proteins usually contain one or more moieties that mediate their recruitment to plasma membranes through protein-protein or protein-lipid interactions. Their multiple functional domains and large molecular size reflect the docking proteins' function as a platform for the assembly of signaling subsystems. Since there have been several excellent general reviews on Gab proteins to date [1–4], here we will focus on the role of Gab docking proteins in cardiovascular and inflammatory disorders.

2. Identification of Gab Family Docking Proteins

Gab1, the first of the three mammalian *gab* genes cloned to date, was originally identified as a Grb2-binding protein from a human glial tumor expression library and found to undergo tyrosine phosphorylation in response to stimulation by epidermal growth factor (EGF) and insulin [5]. It was also isolated as a c-Met-receptor interacting protein in a yeast two-hybrid screen and as the major tyrosine-phosphorylated

protein in cells transformed by the *Tpr-Met* oncogene [6, 7]. Gab2 was cloned as a binding protein and a substrate of the SH2 domain-containing protein tyrosine phosphatase (SHP2) [8–10]. The *Gab3* cDNA was cloned with the aid of the genome sequencing project, using a search strategy based on sequence similarities to Gabl [11]. Although a putative *Gab4* gene has been found in the human genome database, its expression pattern, signaling mechanism, and functional roles have not been characterized to date.

DOS is the only Gab homolog in *Drosophila*. It was identified as a potential substrate for the product of *Corkscrew* (*Csw*) [12], the *Drosophila* SHP2 ortholog, and independently in a screen for mutants that suppress the rough-eye phenotype of a hyper-activated *sevenless* allele [13]. SOC-1, the *C. elegans* homolog, was found in a screen for suppressors of hyperactive Egl-15 (an FGF receptor ortholog) signaling [14].

3. Molecular Structure, Recruitment, and Phosphorylation of Gab Docking Proteins

3.1. Molecular Structure. All Gab docking proteins share a highly conserved N-terminal Pleckstrin homology (PH) domain, proline-rich segments in the central region, and multiple tyrosine residues within the potential binding motifs favored by various SH2 domain-containing signaling proteins (Figure 1) [1–4]. Mutagenesis and *in vitro* binding assays have demonstrated that a number of signaling molecules interact with Gab docking proteins (Figure 1).

3.2. Recruitment. Gab docking proteins utilize several different mechanisms to regulate their subcellular localization. First, the PH domain enables Gab proteins to translocate to plasma membrane patches enriched in phosphatidylinositol 3,4,5-triphosphate (PIP3), a product of phosphatidylinositol-3 kinase (PI3K) [15–18]. Besides the PH domain, Gab docking proteins use at least two additional mechanisms for their recruitment to activated plasma membrane-associated receptors. The first mechanism has been demonstrated only for the interaction between Gabl and c-Met (the receptor for hepatocyte growth factor; HGF) [7]. A region in Gabl (amino acids 450–532), termed the c-Met binding domain (MBD), interacts directly with the tyrosyl-phosphorylated c-Met in response to stimulation with HGF [7, 19–21]. Both the activated kinase domain of c-Met and the MBD in Gabl are involved in this direct interaction [19, 20]. The minimal amino acid sequence sufficient for the direct interaction between Gabl and c-Met, termed the c-Met binding sequence (MBS), consists of 16 amino acids (486–501) [19, 20]. Since no other Gab docking proteins contain the MBS [22, 23], Gab2 interacts with activated receptors via the adaptor protein Grb2, which is also utilized as a secondary mechanism by the c-Met receptor to associate indirectly with Gabl. The importance of this indirect recruitment was revealed in knockout mice expressing a Gabl mutant incapable of binding with Grb2: the phenotype was lethal [21].

3.3. Phosphorylation. Gab-mediated signal transduction is regulated by the site-specific tyrosine phosphorylation of the Gab proteins. Phosphorylated tyrosine residues provide docking sites for the SH2-domains of SHP2, the Crk adaptor protein, phospholipase C (PLC) γ, and the regulatory subunit of PI3K, p85 [2–4]. By recruiting various effectors with SH2 domains, Gab proteins not only promote signal transduction but also translate the receptor-evoked signals into distinct biological properties. Therefore, Gab family proteins function as a signaling platform for an entire signaling subsystem.

The best-characterized effector signaling pathway of Gab proteins is transmitted via the protein tyrosine phosphatase SHP2. SHP2 has two tandem N-terminal SH2 domains, which confer autoinhibition of the C-terminal phosphatase domain [24]. All mammalian Gab proteins, as well as the *Drosophila* DOS and *C. elegans* SOC-1, bind SHP2 (or its homologs), suggesting that the recruitment of SHP2 is an evolutionarily conserved feature of Gab family proteins [24]. Most Gab proteins contain two SHP2 binding sites, which act as a biphosphoryl tyrosine activation motif (BTAM) and bind both SH2 domains, which releases SHP2's autoinhibition [24, 25]. Therefore, SHP2 binding partners, including Gab proteins, may act not only as signaling platforms, but also as allosteric activators.

The functional significance of the Gab-SHP2 interaction has been extensively studied using mutants of Gab family proteins unable to bind SHP2 or its homologs. Mutant DOS bearing a Y to F mutation at either of the two CSW-binding sites is nonfunctional, and Sevenless signaling cannot rescue the lethal phenotype associated with *DOS* loss-of-function mutations [26, 27]. A Gabl mutant that is unable to bind SHP2 fails to transduce the signal for c-Met-dependent morphogenesis in MDCK cells and blocks the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), MAP kinase upon stimulation with epidermal growth factor (EGF), HGF, or lysophosphatidic acid (LPA) [23, 25, 28, 29]. In endothelial cells, the recruitment of SHP2 to Gabl not only regulates vascular endothelial growth factor- (VEGF-) induced migration, but also contributes to HGF-induced migration [30–32]. We also found that the Gabl-SHP2 interaction is involved in the activation of extracellular signal-regulated kinase 5 (ERK5) in gp130-dependent cardiomyocyte hypertrophy [33, 34]. In addition, in certain cellular circumstances, the Gab-SHP2 complex positively regulates other downstream pathways, such as c-Kit-induced Rac activation and β1-integrin-induced PI3K activation [35, 36].

These findings demonstrate that SHP2 is a crucial positive modulator for the activation of ERK1/2. Although the molecular mechanism underlying why the recruitment of SHP2 by Gabl is required for the full activation of ERK1/2 is still not completely understood, two possible mechanisms have been proposed. First, SHP2 may dephosphorylate the recruitment site for the Src-inactivating kinase Csk on the transmembrane glycoprotein PAG/Cbp and paxillin, resulting in the enhanced activation of Src family kinases [37, 38]. Second, SHP2 may dephosphorylate the binding site for p120Ras-GAP on the activated receptors for EGF and on Gabl, thus inactivating the Ras-dependent signaling pathway [37, 39].

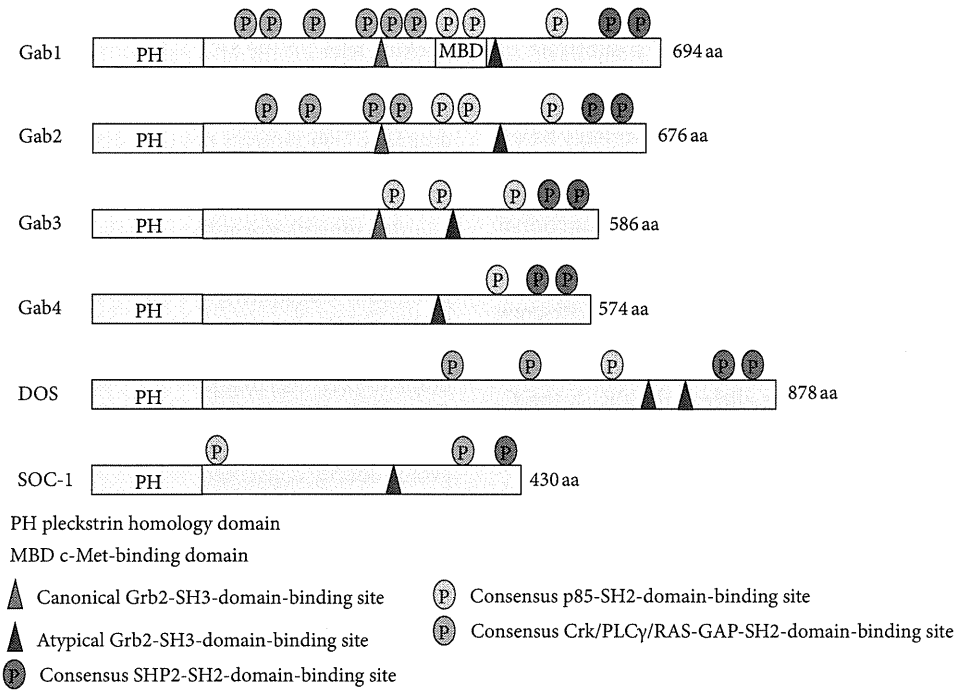

FIGURE 1: Schematic structures of Gab family docking proteins. Shown are the schematic domain structures of three human Gab proteins (Gab1–3), the putative human Gab4 protein, *Drosophila* DOS, and *C. elegans* SOC-1. All Gab proteins consist of a highly conserved N-terminal PH domain that is involved in membrane targeting. The central proline-rich regions mediate the association with SH3 domain-containing adaptor proteins such as Grb2. Consensus binding motifs favored by various SH2 domain-containing proteins such as SHP2, p85, Crk, and PLCγ are indicated.

4. Physiological Functions of Gab Proteins Revealed by Global Knockout or Knock-In Mice

The presence of multiple *gab* genes in mammals suggests that the function of each Gab protein may be specialized or restricted to certain pathways or tissues. On the other hand, these gene products may be functionally redundant. Extensive analyses of the expression level of the *gab* genes by northern blot and RT-PCR have shed some light on this issue [5, 7, 8, 11]. Gab1 shows the broadest expression and greatest abundance: it is found in almost all tissues examined, including the brain, heart, liver, lung, kidney, pancreas, spleen, thymus, and uterus of the adult mouse, and is expressed at early developmental stages, such as in ES cells [11]. Although Gab2's expression is relatively weak in most tissue samples, compared with Gab1, it is abundantly expressed in hematopoietic progenitor cell lines, such as BAF3 and FDC-P1 [5, 7, 8, 11]. The expression of Gab3 is also confined to the hematopoietic system [5, 7, 8, 11]. Thus, the three mammalian *gab* genes have unique but overlapping expression patterns.

Consistent with Gab1's early and broad expression during development, Gab1-knockout ($Gab1^{-/-}$) mice die *in utero* between embryonic days (E) 13.5 and 18.5 with developmental defects in the heart, placenta, skin, and skeletal muscle [40, 41]. In line with the close relationship between Gab1 and c-Met, $Gab1^{-/-}$ mice phenocopy most of the phenotypes of HGF- and c-Met-knockout mice, such as early embryonic lethality with placental defects, reduced liver size, and defects in the migration of muscle precursor cells [40, 41].

Gab1 knock-in mice carrying mutations in the SHP2 binding site show defects in muscle and placental development presumably directed by HGF/c-Met signaling, demonstrating a specific role for the Gab1-SHP2 complex in the migration of muscle progenitor cells [21]. Consistent with these findings, we found that the myogenic differentiation of C2C12 cells induced by IGF-1 or low-serum conditions was strongly enhanced by the adenovirus-mediated overexpression of a mutated Gab1 ($Gab1^{\Delta SHP2}$) incapable of binding SHP2, but inhibited by the overexpression of wild-type Gab1 [42]. This result suggests that Gab1 negatively regulates myogenic differentiation through its association with SHP2. Taken together, these findings suggest that Gab1 plays a key role not only in the inhibition of myogenesis, but also in the maintenance of the undifferentiated state of mesenchymal cells, effected through the activation of SHP2. On the other hand, Gab1 knock-in mice carrying mutations in the p85 binding site show defects in EGF receptor-mediated embryonic eyelid closure and keratinocyte migration [21], and knock-in mice expressing a Gab1 mutant lacking the Grb2 binding sites display an embryonic lethal phenotype and defects in liver, placenta, and craniofacial development [21]. These results support the idea that Gab1 induces different

biological responses through the recruitment of distinct effectors *in vivo*.

In contrast, Gab2-knockout ($Gab2^{-/-}$) mice are viable, generally healthy, and have an apparently normal life span. Although Gab2 was initially believed to be essential for the development of various hematopoietic lineages through its association with SHP2 [43], steady state hematopoiesis is largely normal in $Gab2^{-/-}$ mice [44, 45]. However, $Gab2^{-/-}$ mice exhibit a drastic phenotype in mast cell functioning [44]. Mast cells are major players in allergic responses, and $Gab2^{-/-}$ mice have severe defects in their response to passive allergic challenge; their mast cells display defects in degranulation and cytokine gene expression in response to the activation of FcεRI, the high-affinity IgE receptor. The defective activation of $Gab2^{-/-}$ mast cells is ascribed mainly to their failure to induce PI3K activation. Furthermore, $Gab2^{-/-}$ mice show decreased numbers of mast cells in various tissues, including the skin and stomach, because of weakened c-Kit signaling [45]. These findings suggest that Gab2, which is often upregulated in inflammatory disease, might be an important target for novel therapies against inflammation and allergy [46].

$Gab2^{-/-}$ mice also exhibit an osteopetrotic phenotype that is attributed to the role of Gab2 in regulating RANK- (receptor activator of nuclear factor-κ-B-) dependent signaling [47]. Gab2 associates with RANK and mediates the RANK-induced activation of NF-κB, AKT, and JNK. Bone homeostasis is determined by an intricate balance between the anabolic action of mesenchymal osteoblasts and the catabolic action of osteoclasts. Consistent with Gab2's pivotal role in the differentiation of a variety of hematopoietic lineages [43, 45], $Gab2^{-/-}$ mice exhibit defective osteoclast differentiation, resulting in decreased bone resorption and a subsequent systemic increase in bone mass [47]. In addition, Gab2 has a crucial role in the differentiation of human progenitor cells into osteoclasts [47].

To dissect the Gab2-dependent signaling pathways required for the degranulation of mast cells *in vivo*, Nishida et al. established knock-in mice that express Gab2 mutated at the binding sites for either the PI3K regulatory subunit p85 or SHP2 [48]. They found that both binding sites of Gab2 are required for degranulation and the anaphylaxis response, but not for cytokine production or contact hypersensitivity. Interestingly, the PI3K, but not the SHP2, binding site turned out to be important for granule translocation during degranulation. In particular, the Fyn/Gab2/PI3K-signaling pathway activates a small GTPase, ADP-ribosylation factor (ARF)1, which regulates granule translocation. These results indicated that Fyn/Gab2/PI3K/ARF1-signaling is specifically required for granule translocation and the anaphylaxis response in mast cells [48].

No specific role has been identified to date for Gab3. $Gab3^{-/-}$ mice are healthy and viable, and no obvious phenotype was detected in $Gab3^{-/-}$ macrophages, despite the strong upregulation of this protein during macrophage differentiation [49].

5. Physiological Functions of Gab Proteins Revealed by Conditional Knockout Mice

5.1. The Roles of Gab Proteins in Cardiomyocytes. Because the $Gab1^{-/-}$ phenotype is embryonically lethal in mice, several groups, including ours, have created conditional Gab1-knockout mice, to determine its physiological functions in adulthood [50–53]. Gab1 is exclusively expressed in the heart from E10.5 to 13.5 [40], indicating that it might have a specific role in the heart. Therefore, we created cardiomyocyte-specific Gab1-knockout (Gab1CKO) mice, but these mice are viable and display no obvious cardiac phenotypes [52].

Since Gab1 and Gab2 are expressed in cardiomyocytes, we hypothesized that Gab2 might complement the loss of Gab1. We therefore created cardiomyocyte-specific Gab1/Gab2 double-knockout (DKO) mice by crossing Gab1CKO mice with $Gab2^{-/-}$ mice [52]. Although the DKO mice were viable, they showed a high postnatal mortality rate with marked ventricular dilatation and reduced contractility. In addition, the DKO mice showed remarkable pathological phenotypes including endocardial fibroelastosis and a large number of abnormally dilated coronary vessels in the ventricles. Neuregulin-1 (NRG-1) and ErbB receptors, including ErbB2 and ErbB4, comprise an important signaling pathway for heart development and the maintenance of heart function in adulthood. The NRG-1-induced activation of ERK1/2 and AKT were observed in the hearts of control, Gab1CKO, and $Gab2^{-/-}$ mice, but not of DKO mice. These results suggest that Gab1 and Gab2 share a critically redundant role in NRG-1-dependent signaling in cardiomyocytes (Figure 2).

To determine the effects of the DKO on gene expression, we performed a DNA microarray analysis of cardiac tissues, and found that NRG-1 upregulates the expression of the endothelium-stabilizing factor, angiopoietin-1 (Angl), in the control mice, but not in the DKO mice [52]. Conventional Angl-knockout mice show impaired development of myocardial trabeculae and vessel maturation [54], which are quite similar to the pathological abnormalities in the hearts of the DKO mice. Furthermore, the expression patterns of NRG-1 and ErbB are almost mirrored by those of Angl and Tie2, in the heart, suggesting that these two signaling pathways influence each other like a paracrine signaling circuit in the cardiac microenvironment [55]. These results suggest that the contributions of Gab1 and Gab2 to the crosstalk between NRG-1/ErbB and Angl/Tie2 signaling are required for the maintenance of heart function (Figure 2).

5.2. The Role of Gab1 in Angiogenesis, Vascular Inflammation, and Atherosclerosis. Angiogenesis, the process of new blood vessel formation, is involved in many pathological settings, including ischemia, atherosclerosis, diabetes, and cancer [56]. It has been reported that Gab1 has a role in vascular endothelial growth factor- (VEGF-) dependent signaling in *in vitro* experiments using endothelial cells (ECs) [30, 31]. To reveal the *in vivo* role of Gab proteins in angiogenesis, we created endothelium-specific Gab1 knockout (Gab1ECKO) mice [32]. The Gab1ECKO mice are viable and do not show any obvious defects in vascular development. We then subjected

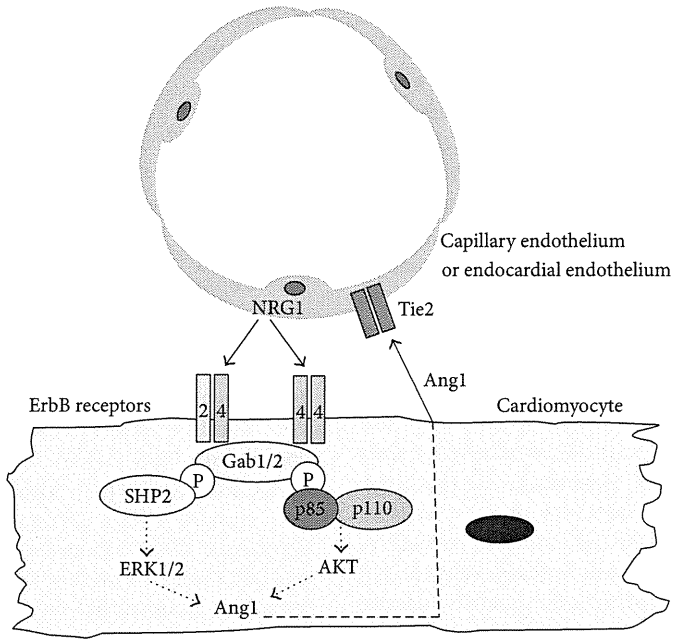

FIGURE 2: Schematic illustration of the roles of Gab docking proteins in the myocardium. Neuregulin-1 (NRG-1) shed from the capillary or endocardial endothelium in the heart activates the ErbB receptors expressed on cardiomyocytes, leading to the tyrosine phosphorylation of Gab1 and Gab2 and subsequent activation of ERK1/2 and AKT. NRG-1/ErbB-Gab1/Gab2 signaling in the myocardium is directly required for the postnatal maintenance of myocardial function. Furthermore, NRG-1/ErbB-Gab1/Gab2 signaling indirectly contributes to the postnatal stabilization of capillary or endocardial endothelium via the upregulation of angiopoietin-1 (Angl). Angl derived from myocardium activates the Tie2 receptor, which is expressed on the cardiac endothelial cells.

Gab1ECKO and Gab2$^{-/-}$ mice to hindlimb ischemia (HLI) induced by unilateral femoral artery ligation. Intriguingly, impaired blood flow recovery and necrosis in the operated limb was observed in all the Gab1ECKO mice, but not in the control (wild-type) or Gab2KO mice. In human ECs, we compared the effects of several angiogenic growth factors and found that HGF induces the most prominent tyrosine phosphorylation of Gab1 and the greatest subsequent complex formation of Gab1 with both SHP2 and p85 [32]. The Gab1-SHP2 complex was required for both the HGF-induced migration and proliferation of ECs via the ERK1/2 pathway and the HGF-induced stabilization of ECs via ERK5. The Gab1-p85 complex also regulated the migration of ECs after HGF stimulation, and it regulates the activation of AKT [32]. A microarray analysis of HGFs effects on gene expression in ECs demonstrated that it upregulates angiogenesis-related genes such as *Kruppel-like factor 2* (*KLF2*) and *early growth response* 1 via the Gab1-SHP2 complex in human ECs (Figure 3) [32]. Furthermore, gene transfer of VEGF, but not HGF, improved the blood flow recovery and ameliorated the limb necrosis after HLI in the Gab1ECKO mice [32]. These results suggest that Gab1 is essential for postnatal angiogenesis after ischemia via PI3K HGF/c-Met signaling (Figure 3).

At the same time as our study, two other groups reported results on postnatal angiogenesis in Gab1ECKO mice using the HLI model [57, 58]. Whereas Zhao et al. reported that endothelial Gab1 is essential for HGF-dependent postnatal angiogenesis, a finding almost identical to ours [58], Lu et al. reported that Gab1 regulates postnatal VEGF-dependent angiogenesis through the protein kinase A- (PKA-) endothelial NOS (eNOS) pathway [57]. Together, these findings provided by three independent groups show that Gab1 is a crucial signal transducer that unites the HGF-dependent and VEGF-dependent signaling and angiogenesis in endothelial cells (Figure 3) [32, 57, 58].

Since the above findings led us to hypothesize that Gab1 might have a role in endothelial homeostasis, we intercrossed the Gab1ECKO mice with apolipoprotein E (ApoE) knockout (ApoEKO) mice. Six-month-old male ApoEKO/Gab1ECKO and littermate control (ApoEKO) mice were treated with angiotensin II (AngII) via an osmotic infusion minipump for 4 weeks. After the AngII treatment, the ApoEKO/Gab1ECKO mice showed significantly exacerbated atherosclerosis and aneurysm formation compared with control mice [59]. The production of proinflammatory cytokines in the aorta was also significantly greater in the ApoEKO/Gab1ECKO than in the control mice. Furthermore, the expression levels of KLF2 and KLF4, key transcription factors for endothelial homeostasis, were significantly reduced in the aortic endothelium of the ApoEKO/Gab1ECKO mice compared with the control mice [59, 60]. Consistent with the reduced expression of KLF2 and KLF4, both vascular cell adhesion molecule-1 (VCAM-1) expression and macrophage infiltration of the aortic walls were enhanced in ApoEKO/Gab1ECKO mice compared with the control mice [59, 60]. Taken together, these findings show that endothelial Gab1 protects

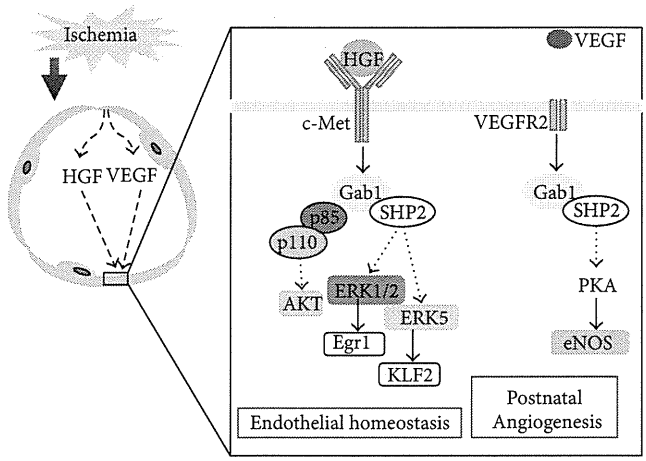

FIGURE 3: Schematic illustration of the role of Gab1 in postnatal angiogenesis and endothelial homeostasis. Hypoxic tissues secrete growth factors, such as HGF and VEGF, which stimulate their specific receptors on endothelial cells (inset). The activation of c-Met receptors leads to the tyrosine phosphorylation of Gab1 and thereby to the subsequent complex formation of Gab1 with both SHP2 and p85. Whereas the formation of the Gab1-SHP2 complex is required for the activation of ERK1/2 and ERK5, the Gab1-p85 complex is essential for the activation of AKT in response to HGF. The ERK1/2 and ERK5 pathways contribute to the upregulation of early growth response 1 (Egr1) and Kruppel-like factor 2 (KLF2). On the other hand, activation of the VEGFR2 receptors leads to the tyrosine phosphorylation of Gab1, and to the subsequent formation of the Gab1-SHP2 complex, which causes the activation of protein kinase A (PKA) and endothelial nitric oxide synthase (eNOS). Collectively, current findings indicate that Gab1 is an essential component of postnatal angiogenesis after ischemia.

the endothelium from AngII-dependent vascular inflammation and atherosclerosis in the *ApoE*-null background, presumably in association with the downregulation of KLF2 and KLF4 [59].

5.3. The Role of Gab1 in Liver Regeneration. Liver regeneration is a rapid and concerted response to injury, in which growth factor-evoked intracellular signals lead to the activation of various transcriptional factors, DNA synthesis, and hepatocyte proliferation. Liver-specific Gab1 knockout (LGKO) mice exhibit defective liver regeneration after a two-thirds partial hepatectomy [50]. The defects in LGKO mice may be ascribed to the decreased proliferation of hepatocytes, due to the decreased activation of ERK1/2 and attenuated upregulation of immediate-early genes, such as c-fos, c-jun, and c-myc, after liver injury [50]. Interestingly, liver-specific SHP2-knockout mice phenocopy the defective liver regeneration of LGKO mice after partial hepatectomy, suggesting that Gab1 plays a critical role in liver regeneration via its association with SHP2 [50]. In addition, Gab1 negatively regulates the hepatic insulin-induced activation of AKT via the ERK1/2-mediated phosphorylation of IRS-1 on Ser612 [51]. Therefore, Gab1 is required not only for liver regeneration but also for the negative regulation of insulin-mediated hepatic glucose homeostasis.

5.4. The Roles of Gab Proteins in Bone Homeostasis. The analysis of $Gab2^{-/-}$ mice shows that Gab2 couples RANK to the downstream signaling essential for osteoclastogenesis, and that Gab2 has a negative regulatory role in osteoblast differentiation [47, 61]. In contrast, osteoblast-specific Gab1-knockout mice display a low-bone-turnover osteopenic phenotype at 2 months of age, demonstrating an essential role for Gab1 in osteoblast functioning [53]. These results indicate that Gab1 and Gab2 have distinct functions in the maintenance of bone homeostasis: Gab1 in osteoblasts and Gab2 in osteoclasts.

6. Gab Proteins in Human Cancers

Gab proteins have been implicated in several hematological neoplasias and solid cancers, although only a few mutations have been reported in human Gab proteins to date. It is currently established that Gab proteins promote tumorigenesis by functioning as "accomplices" of certain oncoproteins or by amplifying signaling upon the Gab proteins' overexpression.

The chromosomal 11q13-14 locus containing the Gab2 gene is amplified in breast, ovarian, and gastric cancers and in acute myeloid leukemia (AML) [62–65]. Gab2 is overexpressed in estrogen receptor-positive cells [66], and a subset of breast cancers is driven by Gab2 overexpression coupled with RTK ErbB2 (also known as Neu or HER2) receptor signaling [62]. Consistent with these clinical results, Neel's group demonstrated that in the cultured human mammary epithelial cell line MCF-10A, the coexpression of wild-type Gab2, but not $Gab2^{\Delta SHP2}$ (incapable of binding SHP2) with ErbB2/Neu/HER2 resulted in an invasive growth phenotype [62]. They also revealed that NeuNT-transgene-evoked mammary tumorigenesis is potentiated in MMTV-Gab2 transgenic mice and attenuated in Gab2-deficient mice [62]. Similarly, Gab2's overexpression can potentiate metastatic melanomas [67]. Furthermore, myeloid progenitors from $Gab2^{-/-}$ mice are resistant to transformation by Bcr-Abl, indicating that Gab2 is required to sustain the leukemogenesis evoked by this oncogenic fusion protein in

a model of chronic myelogenous leukemia (CML) [68]. The phosphorylation of Y177 within the Bcr moiety results in the recruitment of the Grb2-Gab2 complex and the activation of downstream signaling via SHP2 and PI3K, which is essential for the cancer cells' enhanced proliferation and survival [68]. These results suggest that the Grb2-mediated recruitment of Gab2 to the oncogenic fusion protein Bcr-Abl is a critical event for the induction of a CML-like disease. Gab2 is also important in the progression of other hematological neoplasias, such as juvenile myelomonocytic leukemia (JMML), acute myelocytic leukemia (AML), and acute lymphoblastic leukemia (ALL) [65, 69].

That Gab1 plays a role in tumorigenesis is implied by its strong relationship with c-Met receptor signaling, since c-Met is activated, mutated, or overexpressed in a wide range of cancers [19, 70, 71]. Gab1 is also implicated as a mediator of EGFR-signaling-induced tumorigenesis in glioblastomas and intestinal adenomas [72, 73].

The elucidation of this direct linkage between Gab proteins and human cancers may contribute to the development of novel anticancer drugs in the future.

7. Gab Proteins in Human Cardiovascular Diseases

The neuro-cardiofacial-cutaneous (NCFC) syndromes consist of neurofibromatosis (NF), Noonan syndrome (NS), LEOPARD (multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, growth retardation, and sensorineuronal deafness) syndrome (LS), Costello syndrome, and cardiofacial-cutaneous syndrome. All of these syndromes are associated with autosomal-dominant germline mutations within either the core components (Ras, B-Raf, Raf-1, MEK) of the Ras-ERK1/2 pathway or its modulators (NF1, SHP2, SOS, and Spred). The resulting mutant proteins exhibit abnormal activities and disturbed overall fine-tuning of the Ras-ERK1/2 pathway (and to some extent of the Ras-PI3K pathway) [74, 75]. Since the ERK1/2 pathway has a central role in both proliferation and differentiation, many processes in human development and organ maintenance are disturbed by its dysfunction, resulting in various clinical symptoms, such as a distinctive cranio-facial appearance, cardiac defects, musculocutaneous abnormalities, and mental retardation [74, 75]. Germline missense mutations in the SHP2-encoding *PTPN11* gene are seen in approximately 50% of NS cases; this observation contributed to the identification of *PTPN11* as the most common target of somatic mutations in JMML [76, 77]. The most frequent JMML-associated mutation, E76 K, confers an enhanced catalytic activity on SHP2 and requires Gab2 for the transformation of primary murine myeloid progenitors [69]. This result demonstrates that Gab2 is an essential player in JMML and suggests that NS-associated SHP2 mutants may require Gab proteins similarly, as a recruitment tool.

Dominant-negative mutations of SHP2 are reported in LS patients, although NS patients usually carry constitutively active SHP2 mutations [78]. Intriguingly, the expression of LS-associated SHP2 mutants with reduced catalytic activity in cultured cells significantly enhances the EGF-induced association of Gab1 with p85 [79]. This result suggests that LS-associated mutations in SHP2 might potentiate abnormal PI3K activation by blocking SHP2 from dephosphorylating the p85 recruitment sites on the Gab proteins. Collectively, these studies suggest that Gab proteins might exert an important role as "accomplices" of NCFC-associated SHP2 mutants in the pathogenesis of NCFC syndromes.

8. Molecular Mimicry of Gab Proteins by a Bacterial Virulence Factor, CagA

The CagA protein of the gastric pathogen *Helicobacter pylori*, a rod-shaped bacterium that infects the epithelial cells lining the stomach, has been described as functioning as a Gab-like protein [80]. The CagA protein is injected into the cytoplasm of gastric epithelial cells by the bacterium, whereupon it undergoes tyrosine phosphorylation by Src family kinases and c-Abl on E-P-I-Y-A sequence motifs present in its C-terminal region [81, 82]. Subsequently, CagA recruits SH2 domain-containing effector proteins such as SHP2 and Grb2, enabling CagA to effectively take over the signaling pathways that are normally regulated by Gab proteins. This process results in the rearrangement of actin cytoskeleton, cell scattering, and cell elongation, termed the "hummingbird" phenotype, which is reminiscent of the cellular response to Gab activation in cardiomyocytes and other cells [33, 83].

CagA has been categorized as a Gab mimic based on its ability to interact with partners of Gab and exert similar effects in human gastric cells [80]. Intriguingly, this concept of molecular mimicry is strongly supported by transgenic studies in *Drosophila*, demonstrating that a *cagA* transgene can rescue larval viability and photoreceptor development in mutant animals that lack DOS [84]. In addition, an epistasis analysis demonstrated that the complementation of DOS by CagA overexpression requires the expression of the SHP2 ortholog CSW [84]. Thus, these results revealed how CagA can mimic Gab/DOS proteins *in vivo*.

9. Conclusion

Since the discovery of Gab docking proteins, a little more than a decade ago, it has become evident that these proteins play critical roles in a variety of physiological processes as well as in disorders including cancer, inflammation, and cardiovascular diseases. Quite recently, a genome-wide association study conducted by Tamari's group identified Gab1 as a candidate gene for adult asthma in the Japanese population [85]. Whereas the molecular mechanism underlying this association remains unclear, further studies focusing on Gab proteins will aid in elucidating the pathophysiology of this kind of bronchial asthma in the near future. Thus, the versatile functions of Gab docking proteins might extend beyond the original definition of a docking protein. Furthermore, through careful analyses of Gab docking proteins, as shown in this paper, we may be able to obtain a more detailed

understanding of Gab-mediated cardiovascular diseases, cancers, and inflammation.

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Review

Gene and cytokine therapy for heart failure: molecular mechanisms in the improvement of cardiac function

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Nagai T, Komuro I. Gene and cytokine therapy for heart failure: molecular mechanisms in the improvement of cardiac function. *Am J Physiol Heart Circ Physiol* 303: H501–H512, 2012. First published July 9, 2012; doi:10.1152/ajpheart.00130.2012.—Despite significant advances in pharmacological and clinical treatment, heart failure (HF) remains a leading cause of morbidity and mortality worldwide. Many new therapeutic strategies, including cell transplantation, gene delivery, and cytokines or other small molecules, have been explored to treat HF. Recent advancement of our understanding of the molecules that regulate cardiac function uncover many of the therapeutic key molecules to treat HF. Furthermore, a theory of paracrine mechanism, which underlies the beneficial effects of cell therapy, leads us to search novel target molecules for genetic or pharmacological strategy. Gene therapy means delivery of genetic materials into cells to achieve therapeutic effects. Recently, gene transfer technology in the cardiovascular system has been improved and several therapeutic target genes have been started to examine in clinical research, and some of the promising results have been emerged. Among the various bioactive reagents, cytokines such as granulocyte colony-stimulating factor and erythropoietin have been well examined, and a number of clinical trials for acute myocardial infarction and chronic HF have been conducted. Although further research is needed in both preclinical and clinical areas in terms of molecular mechanisms, safety, and efficiency, both gene and cytokine therapy have a great possibility to open the new era of the treatment of HF.

myocardial infarction

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Introduction

Despite significant advances in pharmacological and clinical treatment, heart failure (HF) remains a leading cause of morbidity and mortality worldwide. Numerical evidence suggests that HF is a progressive disorder of which pathogenesis is related to many factors such as ischemia, mechanical stress, inflammation, metabolic disorders, and genetic disorders (74). Many new therapeutic strategies, including cell transplantation, gene delivery, and cytokines or other small molecules, have been explored to treat the various pathophysiological status of HF. Functional improvement after cell transplantation in the failing heart after ischemia has been demonstrated in preclinical and clinical studies for different subsets of bone marrow-derived cells and other adult progenitor cells, as well as cardiac stem cells, embryonic or induced pluripotent stem cells, are considered as cellular sources for generating cardiomyocytes (68, 133). Thus cell therapy is a promising approach for the treatment of HF. One of the most recent hypotheses is that the transplanted cells exert their beneficial effect via a paracrine mechanism in which molecules from transplanted cells may promote angiogenesis, cell survival, stem cell homing, myocardial contraction, and cardiomyogenesis (33). A quantitative proteomic approach and secretome analysis of transplanted cells may enable us to uncover the target molecules for pharmacological or genetic strategy. Since the particular drug or gene therapy may enhance the effect of cell therapy on cardiac regeneration, cell and pharmacological or gene therapy are complementary.

HF is characterized by impaired contraction and relaxation of the affected ventricles. Recently, great attention has been paid to two molecular pathophysiological abnormalities in failing myocytes: downregulation and desensitization of β-adrenergic receptors (β-ARs) (10, 26) and alterations of intracellular Ca^{2+} handling (6, 44). Although treatment with β-blockers improved the prognosis of HF, other pharmacological therapy intended to reverse or bypass β-adrenergic desensitization, such as phosphodiesterase inhibitors, catecholamines, or other positive inotropic agents, showed symptomatic improvement but increased mortality in patients (28). Similarly, transgenic overexpression of β-ARs, G proteins, or protein kinase A (PKA) caused short-term improvements in cardiac function but long-term cardiac dysfunction (23, 26). Ca^{2+} transients in failing cardiomyocytes are characterized by a lower amplitude and slower decline compared with those of normal cardiomyocytes (5, 50). It has been reported that sarcoplasmic reticulum (SR) and its membrane-anchored proteins, ryanodine receptors (RyRs), sarco(endo)plasmic reticu-

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lum Ca^{2+}-ATPase (SERCA), and phospholamban (PLN), play a pivotal role in Ca^{2+}-handling system (5).

Recently, our understanding of the molecules involved in β-adrenergic systems and Ca^{2+} handling has been advanced. Therefore, more appropriate HF therapies by using newly developed drugs or genes has been proposed. Currently, numerous small molecule chemicals that have ability to improve cardiac function or attenuate cardiac remodeling have been reported. These include G protein βγ-subunit (Gβγ) inhibitors, cardiac myosin activators, neuregulin-1β, thyroid hormone analog, and P2X4 purinergic receptor agonists and so on (112, 115). Although a careful elucidation of dose/regimen is required to prevent the potential risk of systemic adverse effects, some of the chemicals have advanced into clinical trials. A phase IIb study of elective allosteric activator of cardiac myosin omecamtiv mecarbil in patients with left ventricular (LV) systolic dysfunction hospitalized for acute HF is now enrolling patients (73). Phase II and phase III studies of a neuregulin-1β fragment and a phase I study of a full-length glycosylated recombinant human neuregulin-1β3 are ongoing (109). Because a complete review of all HF drugs is beyond the scope of this review, the authors recommend recent excellent reviews of key cardiac signaling molecules that are potential drug targets to treat HF (115).

Gene therapy means the delivery of genetic materials into cells to achieve therapeutic effects. As mentioned above, the discovery of new molecular targets for HF therapy facilitate the clinical application of pharmacological manipulations; however, a number of these molecules cannot be intervened systemically, rather suitable for gene therapy. In addition, the heart is easily and selectively accessible by percutaneous catheter approaches so that the myocardium is ready for gene delivery system. Over the past decade, there has been a great advancement in cardiovascular gene transfer technology in terms of the transduction efficiency and safety of viral vectors, methods of vector delivery, and nonviral gene carriers (59, 110, 135). Nonviral vectors such as plasmid DNA, liposome-DNA, and polymer-DNA are less expensive and safe; however, viral vectors are superior to the nonviral vectors in terms of efficiency of myocardial transduction and long-term transgene expression. Currently, the most commonly used viral vector in clinical cardiovascular gene therapy is adenovirus (121). Adeno-associated virus (AAV) and retrovirus are also used in some clinical trials. There are the advantages and limitations in each vector. Recombinant human adenoviruses efficiently deliver and express their genomes in both dividing and nondividing cardiomyocytes. However, the immune response that results in the myocardial inflammation and elimination of infected cells is a large obstacle. AAV can induce stable and long-lasting gene expression compared with adenovirus because of its minimal immunogenecity and particular serotypes, which has excellent tropism for the heart. The major disadvantage of AAV is their limited genome packing capacity. In addition, like adenovirus, the presence of neutralizing antibodies against some serotypes limits the efficiency of gene therapy with AAV. Retrovirus has the advantage in high-transduction efficiency and stable transduction into dividing cells; however, vector genome integration into the host genome raise a concern for the risk of oncogenic transformation. Lentivirus resembles retrovirus in its ability to stably integrate into the target cell genome. In contrast to retrovirus, lentivirus can transduce larger transgenes into nondividing cells. It is possible to minimize the risk of oncogenic transformation by modifying the vector design (75). Because ischemic heart disease is the most common cause of HF, the initial aim of gene therapy was targeted to restore the coronary vasculature. Genetic transfer of angiogenic growth factors or cytokines has been evaluated on animal models, and several controlled clinical trials for ischemic heart disease have been published including VEGF-A165, VEGF-121, VEGF-C, and FGF-4 (66). Along with elucidating multiple steps at which β-AR signaling systems and excitation-contraction coupling are dysregulated, various targets for gene therapy such as G protein-coupled receptor kinase 2 (GRK2), adenylyl cyclase type 6 (AC6), SERCA2a, PLN, inhibitor protein (IN) 1 and 2, and S100A1 have been emerged. Preclinical data suggest that these molecules are important to restore the normal cardiac function. In the first part of this review, we will focus on the recent advancement of the gene therapy targeting on the above seven molecules.

Various growth factors and cytokines, such as granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), and insulin-like growth factor-1 have been reported to prevent cardiomyocytes from apoptosis as well as promote angiogenesis (12, 40, 89). Recently, particular interest has been focused on mesenchymal stem cells (MSCs) from bone marrow or fat tissue as potential cell therapy candidates (130). It has been reported that MSCs secrete a various kind of cytokines, which inhibit apoptosis, inflammatory cascade, and degradation of extracellular matrix, leading to cardiac repair (97). Preclinical studies have shown the improvement in cardiac function after administration of MSC-conditioned medium (33). Secretome analysis of MSCs under stimulated conditions, such as hypoxia or pharmacological and genetic modulation, is expected to unveil novel paracrine factors (97). These findings prompt the clinical trials of cytokine therapy for treating cardiovascular diseases as cell-free therapy alternate to using MSCs. In contrast to newly developed pharmacological chemicals, cytokines are a component of intrinsic factors, and several cytokines have already been used for human hematopoietic or autoimmune diseases, suggesting less concern about safety for clinical application. Therefore, a proper use of cytokines may be a promising therapeutic strategy against HF. In the second part of this review, we focus on the results of recent clinical trials of cytokine therapy, especially of G-CSF and EPO, and introduce the current understanding of their beneficial mechanisms.

Gene Therapy Targeted on β-Adrenergic Signaling

β-ARs are seven transmembrane-spanning G protein-coupled receptors. β-ARs are comprised of three subtypes: β_1-, β_2-, and β_3-ARs. In the heart β_1-ARs are the predominant subtype. Activation of β-ARs in response to sympathetic neurotransmitters such as epinephrine and norepinephrine results in the dissociation of the stimulatory G protein α-subunit (Gαs) from Gβγ. (103). Gαs stimulates AC to produce adenosine 3′,5′-cyclic monophosphate (cAMP) from ATP, leading to the activation of cAMP-dependent PKA, locally bound by an A kinase-anchoring protein. PKA regulates different intracellular, sarcolemmal, and myofibrillar substrates, resulting in the increasing of Ca^{2+} influx and efflux and actin-myosin interaction. Therefore, β_1-ARs play a critical role in the regu-

lation of cardiac chronotropy, inotropy, and lusitropy. Figure 1 shows a schema of β_1-AR signaling pathway and current target of gene therapy in β_1-AR signaling pathway.

G protein-coupled receptor kinase 2. In the failing heart, one of the biological defects is a significant alteration of β-AR system (11). The molecular mechanism of β-AR dysfunction is clarified as a selective reduction of β_1-AR density at the plasma membrane (downregulation) and by an uncoupling of β_1-ARs from G proteins (desensitization) (9, 72). GRK2 are serine/threonine kinases, which consist of a central catalytic domain (~270 amino acids) flanked by an amino-terminal domain (~185 amino acids) and a variable-length carboxyl-terminal domain (~105–230 amino acids). In the heart, GRK2 is a predominant form. When catecholamine occupies β_1-AR and Gα dissociates from Gβγ, the remaining Gβγ facilitates GRK2 translocation and binds to its carboxyl-terminal domain. GRK2 phosphorylates β_1-AR and enhances the affinity for binding to β-arrestins, which prevents further G protein activation and induces the endocytic process of β_1-AR (16, 91). It has been reported that the expression and activity of GRK2 are significantly elevated in human HF (126). Therefore, the potential effects of GRK2 inhibition on in vivo model were extensively studied.

Koch et al. (62) developed carboxy-terminus of β-AR kinase (β-ARK_{ct}) peptide, which competes with endogenous GRK2 for binding to Gβγ and works as a GRK2 inhibitor. Mice overexpressing the β-ARK_{ct} peptide displayed enhanced cardiac contractility in vivo with or without isoproterenol (62). Subsequent studies showed that overexpression of β-ARK_{ct} improves cardiac function in a murine model of HF, such as muscle LIM protein-deficient and calsequestrin-overexpressing mice (41, 102). The beneficial effects of β-ARK_{ct} in HF were also observed in species other than the mouse. Intracoronary delivery of adenovirus encoding β-ARK_{ct} into rabbit HF model after myocardial infarction (MI) revealed the improvement of systolic function and the increase in β-AR density and AC activity (111). Rengo et al. (100) have reported that long-term suppression of GRK2 by delivery of recombinant AAV serotype 6 (AAV6) encoding β-ARK_{ct} improves cardiac contractility and reverses LV remodeling in the rat HF model after MI. Furthermore, adenovirus-mediated β-ARK_{ct} gene transfer in failing human ventricular myocytes increased the contraction and relaxation velocities as well as AC activity in response to β-agonist (131). Recently, Katz et al. (57) have reported successful delivery of self-complementary AAV6 encoding β-ARK_{ct} into the normal sheep heart by using molecular cardiac surgery with recirculating delivery system. The heart treated with AAV6 encoding β-ARK_{ct} showed a significant increase in the maximum values of the first derivative of LV pressure (dP/dt_{max}) compared with control and moderate increase in cAMP and β-AR density (57). Raake et al. (96) delivered AAV6 encoding β-ARK_{ct} by retrograde injection into the anterior interventricular vein of porcine model of HF after MI. Treatment with β-ARK_{ct} significantly ameliorated LV hemodynamics and contractile function in HF pigs (96). Although further studies with preclinical large animal models of HF are needed, β-ARK_{ct} gene therapy in human HF seems to be promising.

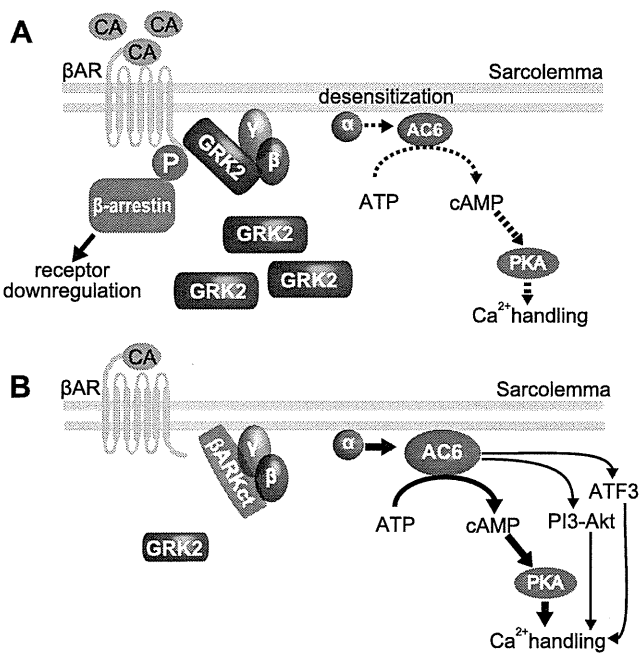

Fig. 1. Cardiomyocyte β-adrenergic receptor (β-AR) signaling during heart failure (*A*) and after treatment with carboxy-terminus of β-AR kinase (β-ARK_{ct}) and adenylyl cyclase type 6 (AC6) gene therapy (*B*). *A*: G protein-coupled receptor kinase 2 (GRK2) phosphorylates β-AR and facilitates the recruitment of β-arrestin, resulting in β-AR downregulation and impairment of further G protein activation. *B*: β-ARK_{ct} inhibits the recruitment of GRK2, resensitizes β-ARs, and promotes normalization of cAMP signaling. AC6 augments Ca^{2+} handling via protein kinase A (PKA)-dependent and -independent pathway. ATP, adenosine 5′-triphosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; CA, catecholamine; PI3, phosphotidylinositol 3; ATF3, activating transcription factor-3.

Adenylyl cyclase type 6. AC6 regulates the conversion of ATP to cAMP, leading to the activation of PKA and initiating a variety of intracellular signaling cascades that influence heart function. The general structure of AC consists of two transmembrane regions and two cytosolic loops. Cytosolic loops comprise the catalytic core, a primary site for the regulation of AC activity. A dominant isoform expressed in mammalian cardiac myocytes is AC6 and AC5 (39, 92). Gao et al. (31) have reported that transgenic mice with cardiac-directed expression of AC6 show normal cAMP production, cardiac function, myocardial β-AR number, and G protein content, expect for an increase in GRK2 content. Under the stimulation through the β-AR, cardiac function and cAMP production were increased (31). It is noteworthy that long-term overexpression of AC6 does not alter β-AR signaling except when receptors are activated. This is in contrast to β-AR and G protein overexpression, which cause detrimental effects on cardiac function. The beneficial effect of cardiac-directed expression of AC6 has been reported in Gαq-associated cardiomyopathy and MI model (105, 118). Roth et al. (106) have reported that indirect intracoronary delivery of adenovirus encoding AC6 produces a significant increase in cardiac contractile responses to β-AR stimulation (106). The same group showed that adenovirus-mediated AC6 expression improved cardiac function in cardiac-directed overexpression of Gαq mice (98). In preclinical studies, by using pig pacing-induced HF model, Lai et al. (65) have reported that intracoronary delivery of adenovirus-encoding AC6 attenuates LV remodeling and increases fractional shortening. LV dP/dt_{max}, the minimum values of the first derivative of LV pressure (dP/dt_{min}),

and cAMP production were enhanced in response to β_1-AR agonist, and levels of B-type natriuretic peptide were reduced (65). Currently, a phase-I/II study of human adenovirus-5 encoding human AC6 gene transfer in patients with HF began enrollment (ClinicalTrials.gov, NCT00787059). The study was a randomized, double-blinded, placebo-controlled study. The vector will be delivered by intracoronary injection with dose escalation. Recently, cAMP-independent effects of AC6 on cardiomyocytes have been reported. These included an increase in activating transcription factor-3 expression, leading to reduced PLN transcription and an increase in phosphatidylinositol 3-kinase (PI3K)/Akt activation, resulting in increased PLN phosphorylation and expression of Bcl-2 protein (32). Although the contribution of these mechanisms is uncertain in the failing human heart, the multifunctional roles strengthen the value of AC6 as the target of gene therapy.

Gene Therapy Targeted on Excitation-Contraction Coupling

The Ca^{2+} handling during contraction and relaxation is a prominent feature of cardiomyocytes (5). In brief, membrane depolarization triggers Ca^{2+} influx through L-type Ca^{2+} channels, followed by a Ca^{2+}-induced Ca^{2+} release through the RyR of SR. The elevated cytosolic Ca^{2+} binds to troponin C and activates the contraction unit. During the systolic period, SR Ca^{2+} content is being depleted, followed by an inactivation of RyRs, and cardiomyocytes then turn from a systolic to diastolic mode. During the diastolic period, the cardiac SERCA2a and sarcolemmal Na^+/Ca^{2+} exchanger are the major mechanisms in Ca^{2+} extrusion. Ca^{2+} uptake through the SERCA pump is negatively regulated by PLN. The amplitude and frequency of the Ca^{2+} transient are regulated by the phosphorylation status of Ca^{2+} cycling regulators, which depends on the balance between the activity of kinase and phosphatase. PKA and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) are essential kinases in cardiomyocytes. PKA is activated by β-AR signal, which is mediated by AC and cAMP formation. PKA phosphorylates L-type Ca^{2+} channels and RyR, which enhances Ca^{2+} influx and promotes SR Ca^{2+} release, respectively. PKA also phosphorylates and inactivates PLN, resulting in the augmentation of SERCA pump activity. Proteins phosphorylated by PKA or CaMK II are actively dephosphorylated by phosphatases such as protein phosphatase (PP) 1 and 2A. Cytosolic PP1 activity is regulated by IN-1 and IN-2, which are activated by PKA phosphorylation and works as an amplifier of β-AR signaling. Figure 2 shows a schema of Ca^{2+} signaling during excitation-contraction coupling and current targets of gene therapy in Ca^{2+} cycling regulators.

SERCA2a. Since Hasenfuss et al. (43) reported that a loss of activity of SERCA2a and subsequent decrease in SR Ca^{2+} uptake are a feature of the failing human heart, the SERCA2a becomes one of the most studied Ca^{2+} handling proteins

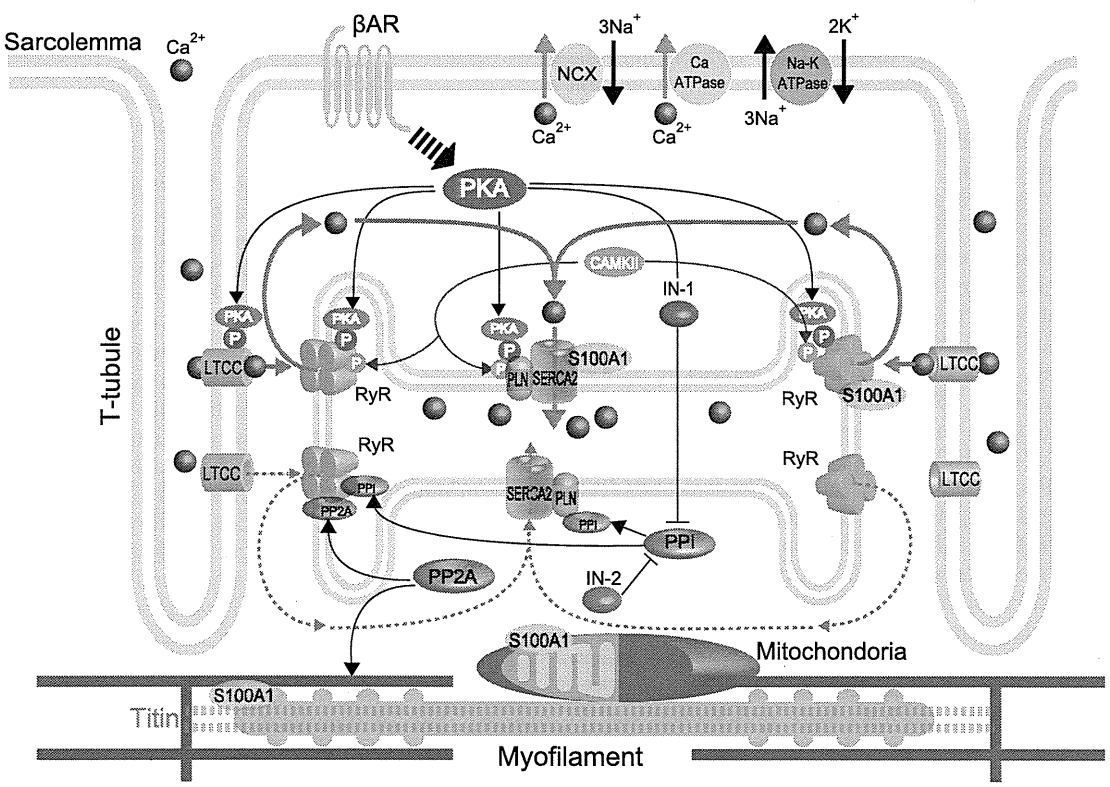

Fig. 2. Target molecules for gene therapy in Ca^{2+} cycling within cardiomyocytes. The amplitude and velocity of the Ca^{2+} transient is regulated by the balance between phosphorylation (P) and dephosphorylation of Ca^{2+} cycling regulators in cardiomyocytes. PKA and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) are major kinases, positively regulating Ca^{2+} cycling. Protein phosphatases (PP) 1 and 2A are major phosphatases, negatively regulating Ca^{2+} cycling. The expected candidate molecules for gene therapy, including sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2A), phospholamban (PLN), inhibitor (IN)-1 and -2, and S100A1 are illustrated. Red arrows indicate the direction of Ca^{2+} movement. Dotted red arrows indicate the decline of Ca^{2+} movement. LTCC, voltage-operated L-type Ca^{2+} channels; NCX, sarcolemmal Na^+/Ca^{2+} exchanger; RyR, ryanodine receptor.

targeting for gene therapy. Overexpression of SERCA2a gene in human failing cardiomyocytes induced a faster contraction and enhanced relaxation velocity (18). The therapeutic potential of SERCA2a gene transfer to failing heart has been evaluated in small animal model of HF. Intracoronary adenovirus-encoding SERCA2a delivery to rats in HF resulting from pressure overload showed improved systolic and diastolic function (19, 107). A direct injection of adenovirus-encoding SERCA2a into myocardium prevented decrease of dP/dt_{max} and dP/dt_{min}, restored wall thickness, and reduced infarct size in rat ischemic-reperfusion model (20). The studies with preclinical large animal models of HF have also proved the therapeutic potential of SERCA2a gene delivery. In a pig model of volume-overload HF due to mitral regurgitation, intracoronary delivery of recombinant AAV1 carrying SERCA2a improved adjusted dP/dt_{max} and LV function (58). In sheep, a HF model induced by MI with a mitral regurgitation model, intracoronary delivery of AAV6 carrying SERCA2a improved dP/dt_{max} and LV remodeling (4). There were two reports, which contradict the above findings. In transgenic rats with cardiac SERCA2a overexpression, there was no beneficial effect on LV function after MI and even an increased mortality due to ventricular arrhythmia (14). Gene transfer of SERCA2a to isolated cardiomyocytes from a canine LV pressure-overload diastolic HF model improved diastolic function, however, but abrogated β-adrenergic responsiveness and inotoropic support (47). Although it has been subsequently reported that proarrhythmic effect is not observed and rather arrhythmias were attenuated in porcine model of ischemia-reperfusion model after SERCA2a gene therapy (95), caution must be taken when gene therapy is applied to HF with normal ejection fraction and MI.

Recently, the first clinical trial of SERCA2a myocardial gene therapy in patients with HF was launched. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease Trial (CUPID) is designed to evaluate the safety profile and the biological effects of gene transfer of the SERCA2a cDNA by intracoronary delivering of AAV1/SERCA2a in patients with advanced HF. This study compares three doses of AAV1/SERCA2a with placebo in patients with advanced HF (New York Heart Administration class III and IV), a LV ejection fraction ≤ 35% of both ischemic and nonischemic dilated cardiomyopathy. In a phase I/II trial, the safety and feasibility of AAV1/SERCA2a administration have been demonstrated (54). Recently, Jessup et al. (55) have reported the results of a phase II trial, in which 39 patients were enrolled. At 6 mo, AAV1/SERCA2a high-dose group showed clinically significant improvements in patients' symptoms and functional status, as well as a significant reduction of cardiovascular events and hospitalization times. This was supported by a reduction in NH_2-terminal prohormone brain natriuretic peptide and improvement of ventricular function/remodeling (55). In this phase II trial, 509 patients were prescreened and 63 patients with undetectable neutralizing antibodies against AAV were considered as candidates because patients who had preexisting anti-AAV neutralizing antibodies did not respond to AAV1/SERCA2a in earlier phase I/II trial. Although a relatively high incidence of the presence of neutralizing antibodies limits the efficacy of gene therapy, SERCA2a gene therapy seems to be promising and the larger trial is awaited.

Phospholamban. In consideration of the good outcomes from a CUPID trial, PLN, a key negative regulator of SERCA2a, also looks like a candidate for therapeutic target of gene therapy for HF. Indeed, transgenic mice overexpressing a mutant PLN superinhibitor of SERCA2a in cardiomyocytes suffered cardiomyopathy (36), whereas PLN knockout mice showed enhanced systolic and diastolic function (71). However, the results of studies, which were designed to rescue HF by ablation of PLN, are variable. Muscle LIM protein-deficient and calsequestrin-overexpressing mice showed the improvement of cardiac function and Ca^{2+} transient when crossbred with PLN knockout mice (77, 108). On the contrary, cardiac ablation of PLN did not rescue the hypertorophic phenotype of β_2-AR overexpressing and mutant myosin-binding protein C mice and did not improve the cardiac dysfunction of mice with cardiac-specific overexpression of tumor necrosis factor-α, although cellular contractility showed significant improvement (29, 53, 116). Adenoviral gene transfer of antisense PLN prevented contractile dysfunction in Ca^{2+} overload-induced LV dysfunction (123). Gene transfer of recombinant AAV encoding phosphomimeric mutant (S16E) form of PLN enhanced myocardial SR Ca^{2+} uptake and suppressed progressive impairment of LV systolic function in BIO14.6 cardiomyopathic hamsters (49). Intracoronary delivery of adenovirus expressing PLN-S16E demonstrated improved cardiac function in sheep pacing-induced HF (60). However, cardiac gene transfer of AAV6 expressing short hairpin RNA against PLN resulted in depressed cardiac function along with reduction of PLN protein (7). These findings suggest that endogenous PLN may play a protective role in the heart in some context. Of note, there are several reports of familial human cardiomyopathies, which are caused by mutations and deletions in the PLN gene or its promoter and where the outcome is a loss of PLN inhibition on SERCA2a (37, 109a). Therefore, before human gene therapy targeting on PLN lowering is launched, more studies on PLN might be warranted.

IN-1 and IN-2. Expression levels and activity of IN-1 and IN-2 are decreased in HF, which is associated with the increase in global and SR-associated PP1 activity, leading to depressed SR Ca^{2+} pump activity (25, 34, 39). Overexpression of IN-1 in cardiomyocytes augmented Ca^{2+} cycling and cell contraction/relaxation in response to β-AR signaling (24). Adenoviral gene delivery of constitutively active IN-1 has been shown to augment cardiac contractility, attenuate hypertrophy, and prevent HF (90). In addition, cardiac-specific overexpression of IN-2 in mouse and gene delivery of IN-2 in cardiomyopathic hamster increased cardiac contractility by augmenting Ca^{2+} cycling (61, 134). However, Wittköpper et al. (132) have recently reported that conditional cardiomyocyte-restricted expression of constitutively active mutant form of IN-1 increases contractile function in the cost of lethal arrhythmia and exaggeration of cardiomyopathy after adrenergic stress and with aging. The authors (132) confirmed similar phenotypes in another line of transgenic mice, which expressed an active form of IN-1 resistant to PKC-α phosphorylation. The authors attributed their results to RyR2 phosphorylation in both of Ser2809 (PKA site) and Ser2815 (CaMKII site) in their transgenic mice. RyR2 phosphorylation in Ser2809 has been previously reported and related to beneficial effects; however, phosphorylation in the CAMKII site has not been examined so far. Phosphorylation of RyR2 at Ser2815 may increase Ca^{2+} fre-