Critical Role for Stromal Interaction Molecule 1 in Cardiac Hypertrophy

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Background—Cardiomyocytes use Ca\(^{2+}\) not only in excitation-contraction coupling but also as a signaling molecule promoting, for example, cardiac hypertrophy. It is largely unclear how Ca\(^{2+}\) triggers signaling in cardiomyocytes in the presence of the rapid and large Ca\(^{2+}\) fluctuations that occur during excitation-contraction coupling. A potential route is store-operated Ca\(^{2+}\) entry, a drug-inducible mechanism for Ca\(^{2+}\) signaling that requires stromal interaction molecule 1 (STIM1). Store-operated Ca\(^{2+}\) entry can also be induced in cardiomyocytes, which prompted us to study STIM1-dependent Ca\(^{2+}\) entry with respect to cardiac hypertrophy in vitro and in vivo.

Methods and Results—Consistent with earlier reports, we found drug-inducible store-operated Ca\(^{2+}\) entry in neonatal rat cardiomyocytes, which was dependent on STIM1. Although this STIM1-dependent, drug-inducible store-operated Ca\(^{2+}\) entry was only marginal in adult cardiomyocytes isolated from control hearts, it increased significantly in cardiomyocytes isolated from adult rats that had developed compensated cardiac hypertrophy after abdominal aortic banding. Moreover, we detected an inwardly rectifying current in hypertrophic cardiomyocytes that occurs under native conditions (ie, in the absence of drug-induced store depletion) and is dependent on STIM1. By manipulating its expression, we found STIM1 to be both sufficient and necessary for cardiomyocyte hypertrophy in vitro and in the adult heart in vivo. Stim1 silencing by adeno-associated viruses of serotype 9–mediated gene transfer protected rats from pressure overload–induced cardiac hypertrophy.

Conclusion—By controlling a previously unrecognized sarcolemmal current, STIM1 promotes cardiac hypertrophy. (Circulation. 2011;124:796-805.)

Key Words: calcium ▪ myocardium, cardiac ▪ hypertrophy ▪ gene therapy ▪ STIM1 ▪ protein ▪ human
triplyphosphate-dependent release of Ca$^{2+}$ from the nuclear envelope and/or CaM-kinase II–dependent activation of protein kinase D has been demonstrated.\cite{6,7} In contrast, it is largely unclear how the CaN-NFAT axis is activated in the presence of the Ca$^{2+}$ fluctuations that occur during cardiomyocyte excitation-contraction coupling.\cite{8} The Ca$^{2+}$ involved here seems to be released independently of the myofilament-activating Ca$^{2+}$ transients. Candidate gates for Ca$^{2+}$ that induce downstream signaling are the LTCC, the ryanodine receptor, or dysfunctional SR Ca$^{2+}$ ATPase (SERCA).

A potential mechanism for Ca$^{2+}$-dependent NFAT activation in cardiomyocytes is store-operated Ca$^{2+}$ entry (SOCE), which has been largely described in nonexcitatory cells but also observed in cardiomyocytes on drug-induced inhibition of the endoplasmic reticulum (ER)/SERCA.\cite{9,10} In nonexcitable cells, 2 groups of proteins, called stromal interaction molecules (STIMs) and Ca$^{2+}$ release–activated channel modulators (ORAs), have been identified as key mediators of SOCE-mediated Ca$^{2+}$ signaling.\cite{11,12} The characterization of STIM1 has unraveled a complex mechanism for Ca$^{2+}$-dependent signal transduction. On depletion of the ER from Ca$^{2+}$, STIM1 forms homo-oligomers and relocates within the ER to regions that are in close vicinity to the cytoplasmic membrane. Thus, STIM1 promotes the opening of ORAI1 in the plasma membrane to induce the entry of extracellular Ca$^{2+}$,\cite{13} resulting in transcriptional activation through NFAT.\cite{14,15} In addition, STIM1 has also been reported to interact with the canonical transient receptor proton channel (TRPC) family in various cell types,\cite{16-20} and contacts between STIM1 and TRPC1 have been mapped in both proteins.\cite{16,21} This is one of the arguments why TRPC channels have likewise been regarded as store-operated channels,\cite{20} despite the fact that they are less selective for Ca$^{2+}$ than ORAI proteins.\cite{22,23} It seems currently unresolved whether store-operated currents activated by STIM1 are based on its interaction with 1 or several ORAI proteins, TRPC proteins, or even a combination of both protein families.\cite{19,24}

Even less is known about the potential function of STIM1 in the heart. Two recent studies carried out in neonatal rat cardiomyocytes (NRCMs) suggested that it translates prohypertrophic stimuli into a growth response,\cite{25,26} but the underlying mechanism remained elusive, and it remained unclear whether STIM-dependent Ca$^{2+}$ entry occurs in the adult heart.

In the present study, we have expanded the experimental scope to cardiomyocytes isolated from an in vivo model for cardiac hypertrophy (transaortic constriction in adult rats) and ultimately to the rat heart in vivo. We analyzed transmembrane currents and intracellular Ca$^{2+}$ concentrations in the presence and absence of drugs that induce SR store depletion and investigated their dependence on expression of STIM.

Our data provide evidence for an inwardly rectifying current that occurs in the absence of store-depleting drugs, which increases significantly in cardiomyocyte hypertrophy and requires STIM1. On the basis of this finding and our finding that reduced expression of STIM1 in vivo partially protects from cardiac hypertrophy, we conclude that STIM1 is a key player in cardiac hypertrophy.
Adenovirus-driven Stim1 overexpression in NRCMs (Figure 1A, red tracing). As detected by immunostaining with an anti-STIM1 antibody, thapsigargin treatment of wild-type rat and mouse cardiomyocytes enhanced the clustering of STIM1 into puncta, a typical phenomenon of SOCE (Figure 1C and Figure IA in the online-only Data Supplement). We also controlled for a potential contribution of noncardiomyocyte cells to these results. Our isolates typically contain <20% of noncardiomyocyte cells, the majority of which are cardiac fibroblasts (Figure IIA in the online-only Data Supplement). Ca2+ influxes in these cells were smaller and not affected by Stim1 silencing (Figure IC in the online-only Data Supplement), excluding a significant contribution of nonmyocyte cells to the above findings. Together, these data recapitulate and expand the existing knowledge that STIM1 is required for SOCE in the presence of an SERCA inhibitor.

We then analyzed whether SOCE likewise exists in adult rat cardiomyocyte and whether it is affected by prohypertrophic conditions. Because Stim1−/− mice die perinatally, a knockdown strategy to reduce expression of Stim1 was chosen. We used a rat model of compensated cardiac hypertrophy resulting from pressure overload by abdominal aortic banding. Sham-operated rats served as controls. Twenty-four days after surgery, both groups were infected with an adenovirus that carries a short hairpin RNA (shRNA) directed against Stim1, in addition to cDNA encoding the dsRed fluorescent protein (Ad-shStim1-dsRed), and adult rat cardiomyocytes were isolated 4 days later (at day 28; Figure 2A). This strategy allowed us to distinguish unaltered from reduced Stim1 expression in cardiomyocytes isolated from the same rat heart. Echocardiography was performed on all animals before adenovirus injection, confirming left ventricular hypertrophy with preserved ejection fraction in the pressure-overloaded group (Table I in the online-only Data Supplement). Suitability of the adenoviral constructs was validated in NRCMs (residual Stim1 mRNA in dsRed-positive cardiomyocytes was 10% to 15% of the mean control value; see also Reference 14). The shStim1 sequence was selected from several sequences tested, which were all found to efficiently prevent hypertrophy of cardiomyocytes (data not shown). Marked differences between cardiomyocytes from adult healthy or hypertrophic rats were observed in patch-clamp recordings of sarcolemmal store-operated currents (I_{SOC}). As depicted in Figure 2B, thapsigargin induced
Figure 2. Stromal interaction molecule 1 (STIM1)–dependent cation currents in adult cardiomyocytes (CM). A, Schematic timescale and experimental strategy to analyze store-operated Ca\(^{2+}\) currents in adult rat cardiomyocytes (ARCMs). Cells were isolated 28 days after abdominal aortic constriction or sham treatment. The Ad-shStim1-dsRed vector was administered on day 24 after surgery. Isolated ARCMs were assigned to the shStim or the control (ie, noninfected) group on the basis of dsRed fluorescence, thus allowing us to compare the effects of reduced and normal Stim1 expression in a collection cells from the same animal.

B, Whole-cell patch-clamp recordings in ARCMs before and after sarcoplasmic reticulum (SR) Ca\(^{2+}\)/H\(^{+}\) store depletion by thapsigargin (TG). Left, Recordings of Ba\(^{2+}\)/H\(^{+}\) current (ISOC) in the presence or absence of thapsigargin-induced SR store depletion in cardiomyocytes from sham-treated rats. Cells in which STIM1 expression had been reduced by Ad-shStim1-dsRed (green symbols) are shown next to tracings of nonsilenced cardiomyocytes (black symbols). Right, The same recordings conducted on ARCMs isolated after pressure overload. Recordings were performed at \(-80\) mV. Analyses are from 3 animals per group with \(10\) cells per animal. The open and filled triangles refer to the corresponding current-to-voltage relations depicted in C. C, ISOC current-to-voltage relation after Ba\(^{2+}\) (asterisk) and Ba\(^{2+}\)/thapsigargin perfusion as indicated in A in noninfected (black) or Ad-shStim1-dsRed–infected (green) ARCMs from pressure-overloaded hearts. Tracings display the average of 12 cells (banding) and 6 cells (banding shStim1). D, Fluorescence analysis of Ca\(^{2+}\) entry in hypertrophic ARCMs. Cardiomyocytes were isolated from hypertrophic hearts as in A and loaded with the Ca\(^{2+}\) sensor Indo-1. Store-operated entry of Ca\(^{2+}\) was induced as described in A and was detected as increased Indo-1 emission ratio (F405/F480). Left, Representative tracings recorded from hypertrophic cardiomyocytes with or without silencing of Stim1. Right, Quantification from 3 independent experiments with 3 animals per group and \(>10\) cells analyzed per animal.
a divalent cation current that was small in cardiomyocytes from sham-treated adult rats but markedly increased in cardiomyocytes isolated from banded adult rats (Figure 2B, black tracings). In both cases, experimentally reduced STIM1 expression dramatically decreased this current (green tracings in Figure 2B). This is further supported by Indo-1 measurement of intracellular Ca\(^{2+}\) on store depletion. Whereas adult rat cardiomyocytes from sham-treated animals allowed only a moderate entry of extracellular Ca\(^{2+}\) after SR depletion (not shown), a strong increase was observed in the analogous experiment with cardiomyocytes from banding-treated animals (Figure 2D, black tracing). Reduced expression of STIM1 potently diminished this response (Figure 2D, green tracing and column).

Caffeine-induced Ca\(^{2+}\) release, which allows the evaluation of SR Ca\(^{2+}\) concentrations, suggested that the different currents of the 4 groups of cardiomyocytes did not originate from different SR Ca\(^{2+}\) loads (Figure IIIB in the online-only Data Supplement). Together, these data demonstrate that STIM1 is crucially involved in thapsigargin-induced SOCE in adult cardiomyocytes.

**Stromal Interaction Molecule 1 Also Promotes a Current in the Absence of Drug-Induced Store Depletion**

Intriguingly, we also observed a current in the absence of drug-induced store depletion. Although marginal in cardiomyocytes from healthy adult rats, this current is clearly visible and enhanced in cardiomyocytes from hypertrophic hearts (Figure 2B; compare the black tracings in the section marked by the empty triangle). This current is completely abolished in shStim1-infected cells from the same cardiomyocyte population (Figure 2B, green tracing in this section) but did not affect excitation contraction coupling because neither cellular Ca\(^{2+}\) transients nor cell shortening was altered in Ad-shStim1-dsRed-infected cells (Figure IIIC and IIID in the online-only Data Supplement). Together, these data demonstrate that STIM1 is crucially involved in thapsigargin-induced SOCE in adult cardiomyocytes.

**Currents recorded in the absence or presence of thapsigargin share ISOC features such as the amplification in a divalent cation–free environment or the inhibition by SKF or by La\(^{3+}\) (Figure IIIA and IIIB in the online-only Data Supplement). In contrast, differences became evident on plotting the current-voltage relation that underlies these currents. Perfusion of hypertrophic adult rat cardiomyocytes with thapsigargin induced a double rectifying current (Figure 2C, black tracing, right panel). The store-operated Ca\(^{2+}\) current was as permeable to barium as to calcium (Figure 2B and Figure IIIB in the online-only Data Supplement).

In contrast to the above, the current-voltage curve recorded in the absence of thapsigargin is predominantly an inward rectifying current (Figure 2C, black tracing, left panel). With Ba\(^{2+}\) (Figure 2B) or a Na\(^{+}\)-free Ca\(^{2+}\) solution (Figure IIIA in the online-only Data Supplement), it developed slowly and reached steady state after \(\approx2\) minutes. This curve shape displays characteristics of gating by ORAI proteins (see the work by Vig et al\(^{28}\)), but the current did not show the pronounced selectivity for Ca\(^{2+}\) over Ba\(^{2+}\) that has been reported for ORAI1 but not for ORAI 2 and ORAI3\(^{29}\) (Figure IIIB in the online-only Data Supplement).

**Effects of Stromal Interaction Molecule 1 Overexpression or Silencing on the Growth of Isolated Cardiomyocytes**

Our finding that STIM1 deficiency reduces sarcolemmal Ca\(^{2+}\) fluxes prompted us to ask whether altered STIM1 expression affects cardiomyocyte growth and intracellular signaling and whether it would involve store-operated channels. Indeed, NRCMs overexpressing STIM1 were significantly larger than LacZ-overexpressing controls, as detected by α-actinin staining and by automated size detection (Figure 3A). In presence of SKF96365, cell sizes were comparable to LacZ controls, suggesting that STIM1 confers hypertrophy through an interaction with ORAI or TRPC proteins. In line with STIM1 being an activator of the NFAT pathway, a reporter assay indicated that STIM1 overexpression enhanced NFAT activity (Figure 3B).

Additional parameters were assessed to test for the functional impact of STIM1 on NFAT activation and hypertrophy. Reduction of Stim1 expression in NRCMs (to \(\approx25\%\) of control; see Figure 3C, left) partially prevented phenylephrine-induced hypertrophy (as determined by size or \(^{3}\)H-leucine incorporation; Figure 3C). Furthermore, NFAT activity and mRNAs encoding atrial natriuretic factor or modulatory calcineurin interacting protein 1 were reduced (Figure 3D). Finally, cellular capacitance as a means of cardiomyocyte size was higher in cardiomyocytes from pressure-overloaded hearts compared with sham, and Stim1 silencing (see also Figure 2A) resulted in significantly lower values (Figure 3E). These data indicate that STIM1 is both sufficient and necessary for the cardiomyocyte hypertrophic response.

**Adeno-Associated Virus-Mediated Silencing of Stromal Interaction Molecule 1 In Vivo Prevents Cardiac Hypertrophy**

We also asked whether endogenous STIM1 levels change under hypertrophic conditions. We found increased amounts of Stim1 mRNA and protein in NRCMs 48 hours after stimulation by endothelin 1 or phenylephrine (Figure IVA in the online-only Data Supplement). Stim1 expression was observed to be moderate in the adult heart (Figure IVB in the online-only Data Supplement) but was significantly upregulated in left ventricular myocardium from rats after pressure overload (Figure IVC in the online-only Data Supplement). To determine the effects of silencing Stim1 on cardiac hypertrophy in vivo, we generated recombinant cardiotropic adeno-associated viruses of serotype 9 (AAV9), allowing for cardiomyocyte-targeted RNAi against Stim1 (AAV9-shStim1) under control of the U6 promoter (Figure V in the online-only Data Supplement). An AAV9 encoding an shRNA directed against luciferase served as a negative control (AAV9-shLuc). Vectors were injected into rats (\(n=9\) per group, \(5\times10^{11}\) genomes per animal), and 28 days later, abdominal aortic constriction was applied (Figure 4A). Eight of 9 animals survived in both groups.

Consistent with the cardiotropic serotype of AAV9,\(^{30}\) cardiac expression of an AAV9–green fluorescent protein...
control construct led to exclusive green fluorescent protein detection in cardiomyocytes, as opposed to endothelial cells or interstitial cells, which we presume to be mainly fibroblasts (Figure VC in the online-only Data Supplement). Furthermore, we determined whether our viral constructs would affect lymphocyte infiltration or capillary density (rather than STIM1 activity in cardiomyocytes). For this, we analyzed tissue sections of rat hearts for the presence of CD8+ and CD45+ cells and for vessels stained positive for von Willebrand factor. Moderately enhanced infiltration by CD45+ lymphocytes and a decrease in the capillary density were observed in hypertrophic hearts but were independent of manipulated Stim1 expression (Figure VI in the online-only Data Supplement).

Reduced Stim1 expression, occurring after infection with AAV9-shStim1 (Figure 4A), significantly prevented cardiac...
hypertrophy, as delineated from serial echocardiographic measurements of left ventricular wall thickness (Figure 4B and Table II in the online-only Data Supplement) and the ratio of left ventricular weight to body weight (Figure 4C). Concordantly, histological analysis showed a reduction in both cardiomyocyte size and myocardial fibrosis in AAV9-shStim1-treated rats (Figure 4C). None of these effects occurred in the control group. Moreover, Stim1-silenced rats exhibited a reduction in Ca\(^{2+}\)/H\(^{+}\) signaling; nuclear translocation of NFATC3 was reduced in cardiomyocytes from Stim1-silenced rats (Figure 4D).

Discussion
The cardiac muscle responds to mechanical and humoral stress by hypertrophic growth of individual myocytes.\(^{31}\) Although some degree of cardiac hypertrophy serves to reduce wall stress and helps to compensate for increased load on the myocardium, sustained prohypertrophic signaling within cardiomyocytes is clearly detrimental and a major factor contributing to the progression to failure.\(^{31,32}\) Cardiac hypertrophy is typically accompanied by activation of Ca\(^{2+}\)/H\(^{+}\)-dependent signaling pathways and reinduction of a fetal gene expression program.\(^{31,33}\)

Among the Ca\(^{2+}\)-dependent signaling pathways that have been implicated in cardiac growth control, calmodulin-dependent activation of the serine-threonine phosphatase calcineurin and subsequent NFAT translocation to the nucleus are particularly important.\(^{34}\) Although disturbances of cardiomyocyte SR Ca\(^{2+}\) release and SR uptake (leading to, for example, an increase in diastolic Ca\(^{2+}\)) clearly become
dominant in more advanced disease stages, calcineurin activation occurs at early stages of cardiac hypertrophy, when excitation-contraction coupling and SR Ca\(^{2+}\) load are still normal and diastolic Ca\(^{2+}\) concentrations are in the physiological range. It is increasingly understood how global impairment of cardiomyocyte Ca\(^{2+}\) handling, as seen in advanced cardiac disease, elicits certain disease-related signaling pathways. In contrast, we know little about the mechanisms that drive the simultaneous activation of various Ca\(^{2+}\)-dependent signaling pathways observed at early disease stages. In contrast to recent advances in understanding inositol triphosphate–mediated Ca\(^{2+}\) release from the nuclear envelope, it remains unclear how activation of the CaN-NFAT axis in cardiac disease occurs.1

In nonexcitable cells, an important mechanism for Ca\(^{2+}\) signaling involves Ca\(^{2+}\) release from the ER and subsequent influx of extracellular Ca\(^{2+}\) into the cytosol. The key protein involved in this SOCE is STIM1, which activates ORAI1, the pore-forming subunit of a Ca\(^{2+}\)-release–activated Ca\(^{2+}\) channel. In contrast to nonexcitable cells, the role of STIM1 in muscle cells is barely understood. Despite the early findings by Hunton et al.9,10 that SOCE also occurs in cardiomyocytes, it remained unclear whether SOCE is causatively involved in cardiac hypertrophy and whether STIM1 ties SOCE to hypertrophy. Although 2 recent reports could show that reduced expression of Stim1 interferes with the response of cardiomyocytes to prohypertrophic receptor agonists, the restriction to neonatal cardiomyocytes and drug-induced SOCE in these studies left unanswered whether SOCE in adult cardiomyocytes indeed triggers disease and whether this involves a Ca\(^{2+}\)-sensing activity of STIM1.

We believe the most important aspects of our study are the identification of a sarcolemmal current in the absence of drug-induced SERCA inhibition, its stronger amplitude in hypertrophic cardiomyocytes, and its dependence on STIM1. Remarkably, a previous study on thapsigargin-induced currents in lymphocytes and Jurkat T cells also reported a current in the absence of SERCA inhibition. Although that current was not the focus of this study, its inward rectifying characteristic shares similarity with the current we found to be STIM1-dependent in cardiomyocytes. The existence of such a current in lymphocytes justifies the presumption that it may likewise depend on STIM1.

A question that remains is whether the STIM1-dependent current in the absence of SERCA inhibition mirrors a true independence from SR calcium store depletion. Opposed to this, one may envision that store depletion also occurs under physiological conditions but is obscured by STIM1-mediated refilling of the SR with calcium. The latter has similarly been proposed to occur in HeLa cells. In contrast, the observation that SR-based Ca\(^{2+}\) stores remain unchanged in cardiac hypertrophy and our finding that silenced STIM1 expression did not alter the SR Ca\(^{2+}\) (Figure IIIC in the online-only Data Supplement) argue against such a scenario. Evidence that STIM1 may indeed function in a store-independent manner comes from studies on arachidonic acid–stimulated Ca\(^{2+}\) signaling in HEK cells. Although there are clear differences with respect to cell type (nonexcitable versus excitable) and receptor activation, STIM1 appears to function independently of ER/SR Ca\(^{2+}\) store depletion in both cases.

To the best of our knowledge, our data are the first to demonstrate a role for STIM1 in cardiomyocytes that is independent of drug-induced store depletion and suggest a critical role for STIM1 in the adult heart. Yet, several important questions remain: If STIM1 functions in the absence of Ca\(^{2+}\) depletion from the SR, what upstream regulatory mechanisms lead to its activation? Furthermore, does STIM1 in this pathway interact exclusively with the recently identified Ca\(^{2+}\) release–activated channels of the ORAI protein family, or are other channel proteins involved? In the years before ORAI1 was discovered, the store-operated Ca\(^{2+}\) channel was expected to be found in another protein family called TRPC. The TRPCs are less selective for cations than ORAI1, a fact that has been cited as evidence that the STIM1-operated channel is ORAI1. However, several studies have shown interactions between STIM1 and TRPC proteins, which justifies the hypothesis that SOCE has >1 origin. Indeed, aside from ORAI1, which was recently proposed to function in cardiac hypertrophy, analogous correlations were established for TRPC1, TRPC3, and TRPC6. Given this, the currents we measured in the presence and absence of SERCA inhibitors deserve a more detailed discussion. Both currents meet criteria of \(I_{\text{SOC}}\) currents, ie, their susceptibility to the channel-blocking drug SKF or to La\(^{3+}\) ions. As in case of the thapsigargin-dependent current, its inability to discriminate between Ba\(^{2+}\) and Ca\(^{2+}\) and double rectification are consistent with currents mediated by TRPC channels, as reported by Yuan et al., Stiber et al., and Huang et al. and in studies that specifically addressed ion gating by TRPC1 or TRPC3 and TRPC6.

On the other hand, the identity of the channel that promoted a current in the absence of thapsigargin raises a new question. Although this current appears to be mainly inward rectifying and thus compliant with ORAI gating, it appears to lack the profound ion selectivity of this channel often stated for this protein family. However, the extent to which ORAI1, ORAI2, and ORAI3 discriminate between cations differs (in descending order). In addition, the cardiac expression of the 3 ORAI isoforms has yet to be determined, and ORAI1 may be functionally replaced by either ORAI2 or ORAI3. Although this supports the idea that the STIM1-dependent current we observe without SERCA inhibition is mediated by one or both of these channel proteins, we may also envisage the participation of heteromeric channels formed by ORAI and TRPC members, as suggested by Liao et al. Future studies should identify the channel that is activated by STIM1 in the absence of SR Ca\(^{2+}\) store depletion, test whether STIM1 oligomerization within the SR and its interaction with plasma membrane ORAI is intact under conditions of cardiac hypertrophy, but also investigate the role of TRPC proteins under such conditions. Interestingly, STIM1 was recently reported to inhibit LTCC in neurons and vascular smooth muscle cells, and the authors of both studies speculate that this mechanism promotes the decision of which Ca\(^{2+}\)-signaling pathways are specifically activated. However, LTCC blockade has been shown to exert an antihypertrophic effect (as opposed to the pro-
hypertrophic role of STIM1) and our in vitro experiments to characterize the STIM-dependent current had been carried out in the presence of an LTCC blocker. This argues against a predominant role of LTCC in STIM1-dependent pro-hypertrophic signaling.

At present, it is unclear whether additional Ca\(^{2+}\)-dependent signaling mechanisms that have been implicated in cardiomyocyte hypertrophy involve STIM1. These include the direct coupling of the CaM-CaN axis to LTCC-induced Ca\(^{2+}\) entry that has been described in neurons\(^{49}\) and the Ca\(^{2+}\) and integrin-binding protein 1 identified as a prohypertrophic calcineurin-interacting protein.\(^{50}\) It remains to be seen whether, and how, STIM1 contributes to these pathways. The answer to these crucial questions will eventually lead to a detailed picture of how STIM1 is activated and what protein it recruits at the plasmalemma to mediate NFAT activation and thereby cardiac hypertrophy in the presence of the large fluctuations of intracellular Ca\(^{2+}\) that occur during excitation-contraction coupling. Taken together, our data demonstrate an important role for STIM1 in the progression of cardiac hypertrophy and suggest a possible role in cardiac disease.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

The cardiac muscle responds to mechanical and humoral stress by hypertrophic growth of individual myocytes. Although some degree of cardiac hypertrophy serves to reduce wall stress and helps to compensate for increased load on the myocardium, sustained prohypertrophic signaling within cardiomyocytes is clearly detrimental and a major factor contributing to the progression to failure. The activation of Ca\(^{2+}\)-dependent signaling pathways has been identified as critical for cardiac hypertrophy. However, it has remained largely unclear how Ca\(^{2+}\) triggers signaling in cardiac myocytes in the presence of the rapid and large Ca\(^{2+}\) fluctuations that occur during excitation contraction coupling. Here, we have studied the role of stromal interaction molecule 1 in cardiomyocytes, a molecule that has been described in several cell types as critical for Ca\(^{2+}\) entry. By manipulating its expression, we found stromal interaction molecule 1 to be both sufficient and necessary for cardiomyocyte hypertrophy in vitro and in the adult heart in vivo. STIM1 silencing by viral gene transfer protected rats from pressure overload–induced cardiac hypertrophy. These data demonstrate an important role for stromal interaction molecule 1 in cardiac hypertrophy and may lead to the development of novel approaches to prevent cardiac dysfunction.
Critical Role for Stromal Interaction Molecule 1 in Cardiac Hypertrophy
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Expanded Methods

Transaortic banding and echocardiographic assessment
Rats were anesthetized for surgery with pentobarbital (65 mg/kg). Lateral laparotomy was performed to expose the suprarenal abdominal aorta and a tantalum clip with an internal diameter of 0.58 mm was placed. Sham-operated rats served as controls and were subjected to the same surgeries except for the creation of the aortic band. Echocardiography was performed every two weeks with a General Electric instrument equipped with a linear 8-14-MHz transducer. The surgeon and echocardiographer were blinded to animal pre-treatment.

Cardiomyocyte isolation for cellular electrophysiology experiments
Isolated adult rat cardiomyocytes were placed in a perfusion chamber on the stage of a LSM510 Meta Zeiss confocal microscope equipped with a 63X water immersion objective (NA: 1.2) and continuously perfused with standard Tyrode solution of the following composition (mM): 135 NaCl; 4 KCl; 1.8 CaCl$_2$; 1 MgCl$_2$; 2 Hepes; 10 glucose; pH 7.4 adjusted with NaOH. Confocal images of transfected cells were obtained by excitation at 568 nm and measuring the emitted light at 585 nm.

Calcium quantitation in cardiomyocytes from adult rats
Cells were loaded for 30 min at room temperature with Indo-1 AM in order to monitor intracellular Ca$^{2+}$. Cells were field stimulated at 0.5 Hz (20 V, 1 ms), and simultaneously illuminated at 305 nm using a xenon arc bulb light. Indo-1 fluorescence emitted at 405 nm and 480 nm were simultaneously recorded using IonOptix acquisition software (IonOptix system, Hilton, USA). Once Ca$^{2+}$ transients’ steady state was reached, stimulation was stopped and standard Tyrode solution was switched to a modified Tyrode containing 40 µM CPA where Na$^+$ was replaced by Li$^+$ and Ca$^{2+}$ was removed to achieve Na$^+$/Ca$^{2+}$ inhibition and complete SR store depletion. Store operated Ca$^{2+}$ entry was determined as an increase of indo-1 fluorescence after adding external Ca$^{2+}$ (1.8 mM) $^5$. SR Ca$^{2+}$ load in single cardiomyocytes was assessed after rapid application of 10 mM caffeine $^6$. 

1
Development and production of recombinant adenoviral and adeno-associated virus vectors

Silencing RNA against STIM1 was designed using the SiSearch program to recognize rat and human STIM1 mRNA sequences. The sense sequence is 5'-GGGAAGACCTCAATTACC-3'. The efficiency and specificity of the sequence was previously validated. Oligonucleotides were annealed and ligated via BamHI/EcoRI into pSIREN-DNR-dsRED express vector using Knockout RNAi Systems technology (Clontech). The recombinant pSIREN-dsRED was then transformed in E. coli, using Fusion-Blue Competent Cells (Clontech). The fragment was inserted via CRE-LoxP site-specific recombination into Adeno-X Viral DNA using Adeno-X Expression System 2 (Clontech). The resulting adenovirus was transfected into HEK293 cells and propagated, thereby generating Ad-shStim1-dsRed and Ad-shScrambled-dsRed as a negative control.

The cDNA for Stim1 (GenBank accession: NM_009287) was cloned by PCR and introduced into the adenoviral vector (pAd-CMV-V5-DEST, Invitrogen) and pT-Rex-DEST30 vector (Invitrogen) by site-specific recombination. A lacZ expressing adenoviral vector (pAd-CMV-V5-LacZ) was used as control.

We used a similar approach to produce self-complementary recombinant AAV9 and kept the same silencing sequence against STIM1. The shStim1 and shLuciferase DNA were cloned to pds-AAV2-EGFP vector (kindly donated by Xiao X, University of Pittsburgh, PA) using MluI and Apal restriction enzymes. For production of AAV2/9-shStim1/Luc viruses, these plasmids were used for cotransfection of 293T cells together with the pDG9 plasmid, encoding the AAV-9 cap sequence, as well as adenoviral helper sequences. For checking of the transfection efficiency, enhanced green fluorescent protein (EGFP) was used under the control of the CMV-enhanced MLC promoter. AAV vectors were produced, purified, and titered using standard procedures. Briefly, 1*10^7 293T cells were prepared one day before the transfection. Totally, 200 µg of DNA (pds-EGFP vector: 50 µg and pDG9 vector: 150 µg) were transfected using the CaCl_2 transfection method. After 72 hr of incubation, cells were harvested and purified by the iodixanol gradient / ultra centrifugation method, and the AAV
fraction was concentrated by the VIVASPIN 20 concentrator (100 kDa cut-off, Satorious, Germany). AAV9-GFP was

**Western blot analysis and immunofluorescence**

For immunofluorescence, the following antibodies were used: anti-STIM1 C-terminal (#S6197, Sigma-Aldrich), anti-α-actinin (#A7811, Sigma-Aldrich) and visualized by applying secondary antibodies directly conjugated to either Alexa Fluor 546 or Cy3 (Jackson).

Protein extraction was performed in lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich) and Western blotting was performed using standard procedures. Protein lysates were electrophoresed on 10% SDS-PAGE gels, transferred onto a nitrocellulose membrane and blocked with 5% non-fat milk for 2 hours at RT. For western blot, the following antibodies were used: anti-STIM1 (#610954, BD transduction laboratories or #4916, Cell Signaling, #S6197, Sigma-Aldrich), anti-NFATc3 (#sc-8321, Santa Cruz), anti-Gß (T-20, #sc-378, Santa Cruz), anti alpha-smooth muscle actin (#ab5694, Abcam) and anti-PP2B (#610259, BD transduction laboratories). A monoclonal anti-glyceraldehyde 3 phosphate dehydrogenase antibody (GAPDH, Chemicon International) was used separately as a loading control. For the analysis of STIM1 distribution, neonatal rat cardiomyocytes isolated by enzymatic digestion were seeded on poly-lysine coated coverslips in MEM containing 1% FCS and cultured for 24 hours. The cells were then cultured in MEM containing 0.1% FCS for another 24 hours. The cells were then kept in Ca²⁺-free HBSS solution for 30-60 min and were later treated with 8 µM thapsigargin for 10 min. The cells were fixed using 4% PFA for 5 min at 4°C. After washing and permeabilisation with PBS and 0.2% TritonX, the coverslips were incubated with primary antibodies against rabbit anti-STIM1 (1:400 diluted in PBS; #S6197, Sigma-Aldrich) and mouse anti-α-actinin (1:1000 diluted in PBS, #A7811, Sigma-Aldrich) for 30 min at 37°C. The corresponding Cy3-conjugated AffiniPure goat anti-rabbit (1:200 diluted in PBS; #111-165-047, Jackson) and Alexa488-conjugated goat anti-mouse IgG (1:200 diluted in PBS) were used as secondary antibodies and added for another incubation for 30 min at 37°C. The coverslips were mounted on slides using Vectashield fluorescence mounting medium containing DAPI. Cy3,
Alexa-488 and DAPI were excited at 453 nm, 488 nm and 405 nm laserlines, respectively. Confocal images were taken using Zeiss LSM 510 META and were processed using Adobe Photoshop software. For TIRF measurements, the coverslips were mounted on slides using PBS. TIRF images were taken using a TILL Photonics iMIC microscope (Graefelfing, Germany) and were processed using ImageJ software.

**CD8 and CD45 Immuno staining**

Twenty minutes after fixation with PFA 4%, heart sections (7 µm) were incubated for 5 minutes with 0.3% hydrogen peroxide in 0.3% serum TBS to avoid endogenous peroxidase activity. After blocking with horse serum, primary antibodies against CD8 and CD45 (AdbSerotec MCA48R and MCA43R respectively) were incubated overnight at 4°C. Vectastain ABC kit (Vector Laboratories BA-2001) was used as secondary antibody, and peroxidase substrate (SigmaFast 3,3’, D4418) was applied on the sections until stain intensity developed. Sections were dehydrated in alcohol and xylene and mounted in cytoseal solution (Richard-Allan Scientific). Pictures were acquired at magnification 20 (Olympus IX71 microscope and CellSense Entry 1.2 software).

**Determination of capillary density**

Eight µm cryosections of sham, AAV9-shLuciferase and shStim1 rats hearts were permeabilized with Triton 0.3% and then blocked with BSA 5% for 30 minutes each. Double immunolabeling allowed the identification of vessels with von Willebrand Factor antibody (#A0082, Dako) and cardiomyocytes by vinculin antibody (#V9131, Sigma-Aldrich)⁸,⁹. Both antibodies were incubated overnight at 4°C. Incubation with appropriate secondary antibodies (Invitrogen) was 1 hour at room temperature before staining with DAPI and mounting of the sections in Mowiol (Polysciences, Inc). A minimum of 10 fields / section was recorded at x20 (Olympus IX71 microscope and CellSense Entry 1.2 software). For each section, the number of cardiomyocytes and capillaries was determined using ImageJ software.
Cellular tropism of rAAV9 vectors after intravenous injection

Four rats (2 months, 300 grams) were transduced either with a vector rAAV90GFP that expresses the marker protein GFP or with saline. Vectors were administered by tail vein injection. One month after injection, rats were sacrificed and tissue was fixed by perfusion fixation with PFA 4% solution. Hearts were then removed, immersed in PFA 4% overnight and then in sucrose 15% and 30% for at least 4 hours each before embedding in OCT and storage at -80°C. Eight µm cryosections of these hearts were permeabilized with Triton 0.3% during 30 minutes and then blocked with BSA 5% for 30 minutes. Immunolabeling was performed using an α-actinin (Sigma-Aldrich) and appropriate secondary antibody (Invitrogen) before staining with DAPI and mounting in Mowiol. Images were taken using a confocal microscope (LSM 510 META, Zeiss, Germany) and were processed using Adobe Photoshop software.

Experiments in neonatal cardiomyocytes and cardiac fibroblasts

Neonatal rat cardiomyocytes were isolated according to standard procedures [10]. Whole hearts from newborn rats were excised and were transferred into Ca^{2+} and bicarbonate-free HEPES-buffered Hanks’ medium (HBSS). The hearts were then cut into pieces and digested with trypsin (#215240, Becton Dickinson) under constant stirring. The primary cells that were collected after passing through a 40 µm cell strainer were seeded in uncoated plastic dishes for 1 hour at 37°C / 1% CO_2. The supernatant containing the cardiomyocytes was collected and CM were cultured in MEM containing vitamin B12, NaHCO_3, BrdU and 1% FCS. These were almost exclusively cardiomyocytes that stained positive for α-actinin (>95%). The plastic dishes containing the cardiac fibroblasts were washed with PBS and cells were cultured in MEM containing vitamin B12, NaHCO_3 and 1% FCS. For the hypertrophy assay, NRCMs were plated in 1% FCS for 24 hours. The cells were infected with respective adenoviruses and were incubated for a further 24 hours in 1% FCS. The medium was then changed to 0.1% FCS and after 24 hours, were stimulated with 50 µM phenylephrine (PE) in MEM containing 0.1% FCS for 48 hours. Unstimulated control cells
were incubated in NRCM with 0.1% FCS. Protein synthesis was assessed by $^3$H-leucine incorporation as previously described\textsuperscript{11}. To determine cardiomyocyte area after Stim silencing, thirty individual $\alpha$-actinin-stained cardiomyocytes in 10 fields were examined in each condition in triplicate. ANF and MCIP1 mRNA expression was determined by quantitative RT-PCR as described previously\textsuperscript{7}. NFAT activity was detected by luminescence using a luciferase reporter construct containing repetitive NFAT recognition sites (gift from J. Molkentin, University of Cincinnati). For the proliferation assay, neonatal cardiac fibroblasts were seeded in 1% FCS. After 24 hours, they were infected with adenoviruses. After 24 hours of infection, the cells were stimulated with 10% FCS and cultured for 48 hours. Unstimulated control cells were incubated with 1% FCS.

**Ca$^{2+}$ measurements in neonatal mouse cardiomyocytes**

Cardiomyocytes from neonatal wildtype or Stim$^{1/-}$ mice (see below) isolated by enzymatic digestion were cultured in a humidified 37° C/1% CO$_2$ incubator for 48 h. Prior to the quantitation of Ca$^{2+}$, cells were incubated with 2 $\mu$M Fura-2 for 45 min at 37° C, followed by washing in HBSS buffer without Ca$^{2+}$ and bicarbonate for 30-60 min at RT. After taking measurements for 1 min, 8 $\mu$M thapsigargin (Alexis Biochemicals) was added. 2 mM CaCl$_2$ was added after 2.5 min and 5 min, respectively. During the entire procedure, cells were imaged through a 100X objective with dual excitation at 340 nm and 380 nm, and monitored at 510 nm. The background fluorescence correction was made and the ratio F340/F380 was determined.

**References for Expanded Methods section**


**Supplemental table 1.** Echocardiographic characterisation of rats at day 23 after aortic banding and before Adv-shStim1-dsRed administration (see Figure 2A).

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<td>LVPW (mm)</td>
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<td>Fractional shortening (%)</td>
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**Supplemental table 2.** Echocardiographic characterisation of sham of AAV9-shLuc or AAV9-shStim1 treated rats at day 28 after aortic constriction (see Figure 4A).

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P-values are for comparison between AAV9-shLuc and AAV9-shStim1
Supplemental Figure 1. Detection of endogenous STIM1 in cardiomyocytes. 

(A) Detection of endogenous STIM1 in isolated neonatal mouse cardiomyocytes. Cardiomyocytes from Stim1−/− mice served as negative control. Immunofluorescent staining for STIM1 using a C-terminal antibody (red) and for α-actinin (green). Nuclei were stained with DAPI. Scale bar represents 10 μm.

(B) Detection of endogenous STIM1 in isolated neonatal rat cardiomyocytes. Immunofluorescent staining for STIM1 using an N-terminal antibody (red) and for α-actinin (green). Nuclei were stained with DAPI. Scale bar represents 10 μm.
Supplemental Figure 2.

(A) Relative percentage of cardiomyocytes (CM), cardiac fibroblasts (CF) and non-CMs/non-CFs (Others) in the respective fractions isolated from neonatal rat hearts.

(B) Western blot detecting STIM1 in cardiac fibroblasts 72 hours after infection with Ad-shStim1 (left) and with Ad-Stim1 (right) and quantitative analysis of the results.

(C) Fluorescence analysis of Ca²⁺ entry in neonatal rat cardiac fibroblasts. Left, representative tracings of the Fura-2 emission ratio in CFs infected with Ad-Stim1 (red) and Ad-shStim1 (green). Arrow indicates addition of thapsigargin. Silencing STIM1 did not influence SOC entry (Ad-Stim1 n=63; Ad-LacZ n=56; Ad-shStim1 n=7; Ad-shScr n=14). Right, quantitative analysis of the experiments.

(D) Proliferation assay on fibroblasts infected with Ad-Stim1 (red) or Ad-shStim1 (green) and stimulated for 48 hours with 10%FCS. Data are mean ± s.e.m., and * P<0.05, ** P<0.01 and *** P<0.001.

Supplemental Figure 2. Purity of cardiac fibroblast and cardiomyocyte preparations and cardiac fibroblast SOC and cell proliferation.
Supplemental Figure 3. Cardiomyocyte transmembrane current characteristics, SR Ca2+ content and contractility.

(A and B) Current density curves and quantifications recorded from CM of hypertrophied hearts, without (A) or with (B) drug-induced Ca2+ store depletion. Recordings were taken at -80 mV in varying ionic conditions with/without the SERCA-inhibitor thapsigargin.

(A) Response in the absence of thapsigargin. Upon replacing Na+ in the external solution by NMDG, an inward rectifying current developed slowly and reached steady state after ~2 min. The current developed similarly when Ba2+ (90 ± 34%) was used as a surrogate for Ca2+ (see also B). Further replacement by a divalent cation-free (DVF) solution elicited a change in current amplitude (345 ± 36%), caused by the penetration of monovalent cations through the plasma membrane. This current was inhibited by La3+ (100 μM) or likewise by SKF966566 (10 μM), indicating that ORAI or TRP channels are involved.

(B) Response in the presence of thapsigargin. When added at steady-state in sodium-free, calcium-containing medium (Ca2+/NMDG), thapsigargin-induced depletion of SR-based Ca2+ caused Ca2+ entry through the plasma membrane. A SOC current was also observed with Ba2+ (112 ± 12% of Ca2+), which is consistent with the ion selectivity profile of SOC channels. Exchange to DVF solution elicited a strong amplitude that is fully revertable by La3+, again indicating that ORAI or TRP channels conferred this current.

(C) Analysis of Ca2+ transients after loading of cells with Indo-1 and detection of the emission ratio F405/F480. The mean amplitude of Ca2+ transients after caffeine administration is shown. n=12 cells per group from n=3 hearts each.

(D) Fractional cell shortening in Ad-shStim1-dsRed and non-infected cardiomyocytes isolated from sham-operated rats or from rats with pressure overload-induced cardiac hypertrophy.

* indicates statistically significant difference compared to control.
Supplemental Figure 4. Upregulation of STIM in cardiac hypertrophy.

(A) Left, western blot for STIM1 in neonatal rat ventricular myocytes stimulated for 48 hours with the prohypertrophic agents phenylephrine (PE, 50 μM) or endothelin 1 (ET1, 100 nM). Right, quantification of the western blot results. Data are from 3 independent experiments.

(B) Left, western blot for STIM1 in isolated adult rat cardiomyocytes and left ventricular myocardium. Thymus served as a control. Alpha-smooth muscle actin staining was used to assess the purity of cardiomyocyte preparation and the absence of contamination with smooth muscle cells. Right, quantification of the western blot results. N=2 animals per group in duplicate.

(C) Left, western blot for STIM1 in rat heart lysates 14 and 28 days after aortic constriction or sham treatment (28 days after surgery). Echocardiographic measurements showed the development of compensated hypertrophy in banded-animals (LV anterior wall: 1.6±0.2mm in Sham vs. 2.2±0.2mm at day 14 and 2.9±0.1mm and day 28; Fractional shortening: 57±2% in Sham vs. 77±1% at day 14 and 77±5% at day 28). Right, quantification of the western blot results. N=6 animals per experiment per group.
Supplemental Figure 5. Generation of recombinant AAV9 vectors and analysis of cell tropism.

(A) Partial map of the adeno-associated vector used to express a short hairpin RNA against Stim1. Self-complementary double-stranded adeno-associated virus genome (AAV2) was pseudotyped into the AAV9 capsid. The shLuc construct was generated using the same methodology.

(B) Western blot showing the dose-dependent silencing of Stim1 in NRCM 10 days after infection with AAV9-shStim1 compared to AAV9-shLuc.

(C) Cardiomyocyte tropism of AAV9. Fluorescence detection of PFA (4%) fixed cryostat tissue sections (8 μm) of rat left ventricular tissue 30 days after injection with or without rAAV9-GFP. rAAV9-GFP treatment result in strong GFP expression in cardiomyocytes but not in non-cardiomyocyte interstitial cells or vascular cells.
Supplemental Figure 6. Assessment of lymphocyte infiltration and capillary density.

(A) Sections of left ventricular tissue from AAV9-shLuc and AAV9-shStim1 treated rats and from control rats stained for CD8 (left panel) and CD45 (right panel). Spleens from normal rats were used as positive controls.

(B) Quantitative comparison of the CD8-stained or CD45-stained area between the four groups. Quantification was performed using ImageJ software. n=3 animals per group.

(C) Mean total capillary density and capillary on cardiomyocyte ratio in the endomyocardium of the left ventricle. N=30 fields per group from n=3 animals each.