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Rapid communication

Orai1 and Stim1 regulate normal and hypertrophic growth in cardiomyocytes

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ABSTRACT

Cardiac hypertrophy is an independent risk for heart failure (HF) and sudden death. Deciphering signalling pathways dependent on extracellular calcium (Ca^{2+}) influx that control normal and pathological cardiac growth may enable identification of novel therapeutic targets. The objective of the present study is to determine the role of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel Orai1 and stromal interaction molecule 1 (Stim1) in postnatal cardiomyocyte store operated Ca^{2+} entry (SOCE) and impact on normal and hypertrophic postnatal cardiomyocyte growth. Employing a combination of siRNA-mediated gene silencing, cultured neonatal rat ventricular cardiomyocytes together with indirect immunofluorescence, epifluorescent Ca^{2+} imaging and site-specific protein phosphorylation and real-time mRNA expression analysis, we show for the first time that both Orai1 and Stim1 are present in cardiomyocytes and required for SOCE due to intracellular Ca^{2+} store depletion by thapsigargin. Stim1-KD but not Orai1-KD significantly decreased diastolic Ca^{2+} levels and caffeine-releasable Ca^{2+} from the sarcoplasmic reticulum (SR). Conversely, Orai1-KD but not Stim1-KD significantly diminished basal NRCM cell size, *anp* and *bnp* mRNA levels and activity of the calcineurin (CnA) signalling pathway although diminishing both Orai1 and Stim1 proteins similarly attenuated calmodulin kinase II (CamKII) and ERK1/2 activity under basal conditions. Both Orai1- and Stim1-KD completely abrogated phenylephrine (PE) mediated hypertrophic NRCM growth and enhanced natriuretic factor expression by inhibiting G_q-protein conveyed activation of the CamKII and ERK1/2 signalling pathway. Interestingly, only Orai1-KD but not Stim1-KD prevented Gq-mediated CaN-dependent prohypertrophic signalling. This study shows for the first time that both Orai1 and Stim1 have a key role in cardiomyocyte SOCE regulating both normal and hypertrophic postnatal cardiac growth in vitro.

1. Introduction

Cardiac myocytes express a large number of Ca^{2+} signalling systems that are required to regulate many different cellular functions including physiological growth, contractile function and pathological hypertrophy [1]. Sustained Ca^{2+} signals are required to induce physiological postnatal and pathological cardiac hypertrophy via the CamK and the calcineurin-NFAT pathway, which are separate from the Ca^{2+} signals required for electric contraction coupling (ECC) [2].

Store operated Ca^{2+} entry (SOCE) is an important process in cellular physiology that controls such diverse functions as refilling of intracellular Ca^{2+} stores, activation of enzymatic activity and gene transcription. Recently the two key players of SOCE have been identified, Stim1 and Orai1. In several non-excitable cells but also in skeletal and smooth muscle cells it was shown that Stim1/Orai1 is responsible for SOCE [3]. Moreover, a recent study demonstrated for the first time the expression of Stim1 in cardiomyocytes and investigated a role of Stim1 in cardiac hypertrophy [4].

Stim1 is a 77 kDa single-pass transmembrane protein located primarily in the ER membrane. Stim1 proteins possess conserved N-terminal Ca^{2+} binding EF hands within the ER lumen, where they are thought to sense the luminal Ca^{2+} concentration. Orai1 is a small plasmamembrane protein of 32 kDa with four transmembrane domains and an amino carboxyl end that face the cytosol [5]. Orai1 was identified as the CRAC (Ca^{2+} release-activated Ca^{2+}) channel pore-forming subunit protein. Ca^{2+} release by IP-3 Receptors causes Stim1 oligomerization and relocalisation into ER-plasma membrane junctions. This subsequently activates Orai1 in the adjacent plasma membrane and causes Ca^{2+} entry into the cytosol via CRAC that mediates Ca^{2+} signals required for activation of CamK and CnA.

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However the exact function of Orai1 and Stim1 in cardiac muscle is unknown. In the present study we describe the existence of a Stim1/Orai1 signalling system in cardiac myocytes and provide evidence that both Orai1 and Stim1 have a key role in cardiomyocyte SOCE regulating both normal and hypertrophic postnatal cardiac growth in vitro.

2. Materials and methods

2.1. Isolation and primary culture of neonatal rat ventricular cardiomyocytes

Ventricular cardiomyocytes from 1 to 2 day old rat neonatal hearts (NRCMs) were prepared by trypsin digestion as described previously [6]. After 24 h the medium was replaced by a 0.5% serum-containing DMEM and NRCM were transfected with different siRNAs. 48 h after transfection cells were stimulated with phenylephrine for up to 48 h. After indicated time points, cells were washed with PBS and either subjected to TRIZOL or lysis buffer (PBS pH 7.4, SDS 2%, 2 mM EGTA/EDTA) containing a mixture of 1% v/v phosphatase inhibitors. In a subset of experiments NRCMs transfected with the different siRNAs were transfected on the same day with a EGFP-NFAT adenovirus and the cellular localisation of EGFP-NFAT was analysed 48 h after transfection and 24 h after treatment with phenylephrine.

2.2. small RNA interference

Custom-designed synthetic Stim1 and Orai1 small interfering RNA (siRNA) and scrambled siRNA as a negative control were purchased from Eurogentec. NRCMs were transfected with Stim1, Orai1 and control siRNA oligonucleotides (5 nM) by using HiPerfect transfection reagent according to the manufacturer's instructions (QIAGEN). A custom-designed synthetic scramble siRNA coupled with FITC was purchased from Applied Biosystems and was used to measure the efficiency of the transfection.

2.3. Indirect immunofluorescence

Imaging of NRCMs was carried out as described previously [6] and NRCMs grown on glass coverslips were treated with Stim1, Orai1 or scrambled as described above. After 48 h cells were fixed, permeabilized and labeled with antibodies specific to Stim1, Orai1 and sarcomeric actinin followed by the corresponding Cy3-conjugated, Cy5-conjugated or FITC-conjugated secondary antibodies (Jackson ImmunoResearch Lab) (1/100). Confocal images (CLSM) were obtained using a 63× water objective on a Leica TCS SP laser scanning confocal microscope. Digitized confocal images were processed by Adobe Photoshop.

2.4. Intracellular Ca^{2+}-measurements

Intracellular Ca^{2+}-transients of Fura 2-AM loaded (2 μmol/L) NRCM were obtained 96 h after treatment with siRNA. Cells were electrically stimulated with a biphasic pulse to contract at 37 °C at 1 Hz and excited at 340/380 nm. Fluorescence emission was detected at 510 nm, digitized, and analysed with T.I.L.L.VISION software (v. 3.3). In a subset of experiments, the total SR Ca^{2+} content was immediately assessed after termination of Ca^{2+} transient measurements. After 2 min of electrical stimulation (2 Hz), myocytes were abruptly exposed to $0Na^{+}/0$ Ca^{2+}solutions with caffeine (20 mM). The peak of the caffeine-induced Ca^{2+} transient was used as an index of the SR Ca^{2+} load. For SOCE measurements SR Ca^{2+} stores were depleted by multiple applications of caffeine (10 mM) in conjunction with the SERCA inhibitor thapsigargin (1 μM) to prevent store refilling and achieve maximal store depletion. Verapamil was added to prevent L-type Ca^{2+} entry (10 μM) to the solutions. Relative changes in intracellular Ca^{2+} were monitored following reperfusion of 2 mM Ca^{2+} Ringer solution. In a subset of experiments cells were stimulated with phenylephrine 30 min before the SOCE measurements.

For recording of spontaneous Ca^{2+} oscillations intact NRCMs were loaded with 5 μM Fluo3-AM for 30 min. Confocal images of Fluo3-AM fluorescence (excitation at 488 nm and emission detection >515 nm) were obtained on a Leica TCS SP laser scanning confocal microscope with a 20× water immersion objective. Time-lapsed (xy, 200 ms/frame) were obtained.

Spontaneous Ca^{2+} oscillations were counted in the different conditions and were compared directly.

2.5. Western blotting

Western blotting was performed as previously [7] reported to assess cardiac protein levels of Stim1, Orai1, calsequestrin (CSQ), total PLB, Phospho-PLB-Threonine 17, ERK, Phospho-ERK. After probing with a corresponding pair of Alexa Fluor 680- (Molecular Probes; 1:20,000) and IRDye 800CW-coupled (Rockland Inc.; 1:20,000) secondary antibody, respectively, proteins were visualized with a LI-COR infrared imager (Odyssey. Signals were normalized to CSQ densitometric levels.

2.6. RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA isolation from neonatal cardiac myocytes cultures was performed applying the TRIZOL method, according to the manufacturer's protocol (Invitrogen) as previously described [7]. First strand cDNA synthesis from 1 μg of total RNA was carried out by the use of the iScript cDNA Synthesis Kit (BioRad). Quantitative PCR was carried out on a MyiQ Single-Color Real-Time PCR detection system (BioRad) for murine and rat 18s, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), Stim1, Stim2, Orai1, Orai2, Orai3, and MCIP1.4.

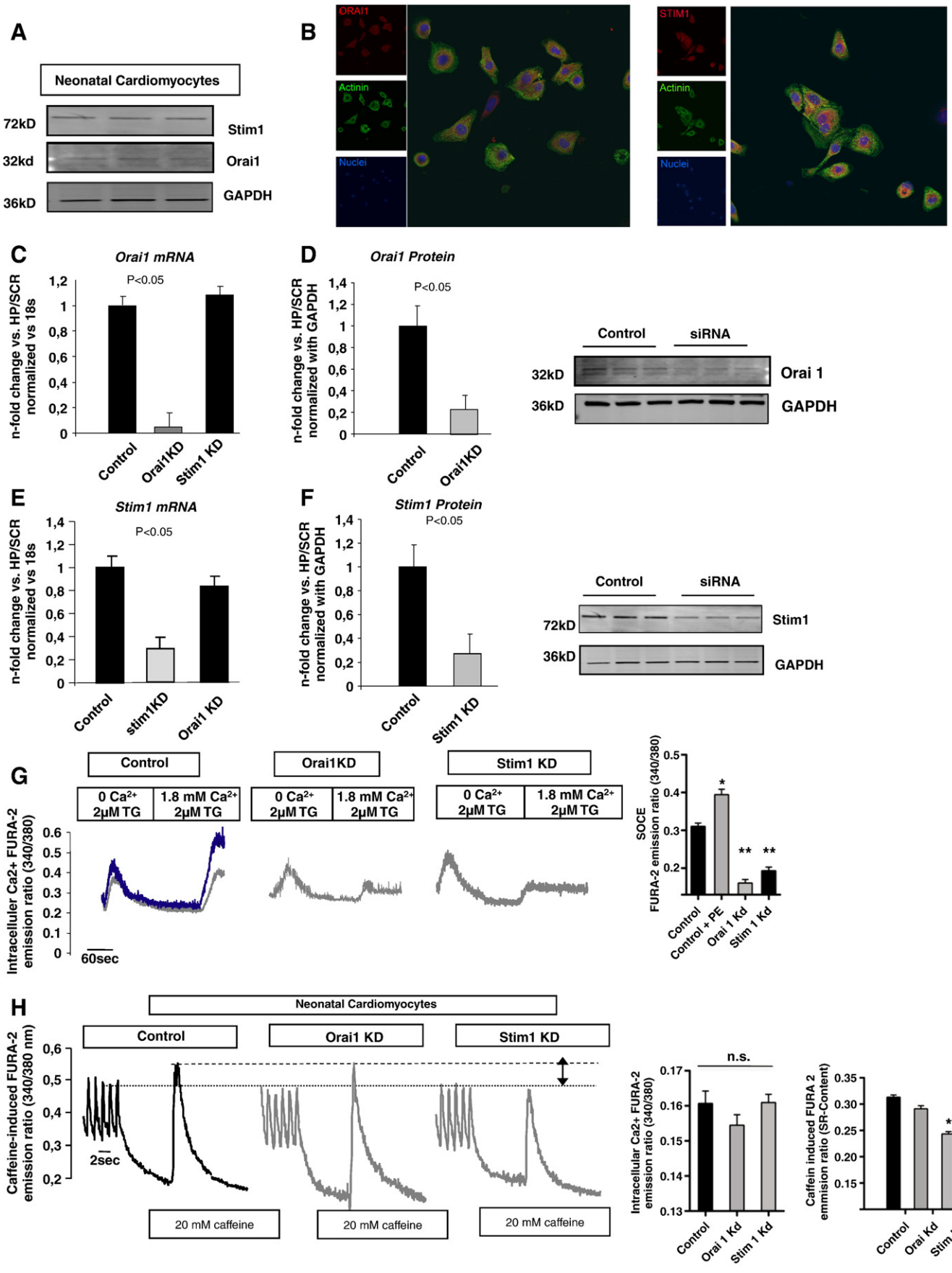
2.7. Statistics

Data are generally expressed as mean ± SEM. An unpaired two-tail Student's *t*-test and two-way repeated measures ANOVA were performed for statistical comparisons. For all tests, a p value of <0.05 was considered as significant.

3. Results and discussion

Since Orai1 and Stim1 orchestrate Ca^{2+}-dependent regulation of skeletal muscle growth and key biological functions in non-cardiac

Fig. 1. Stim1 and Orai1 are expressed and are required for SOCE in neonatal and cardiac myocytes. (A) Immunoblotting for Stim1 and Orai1 in cultured neonatal and adult cardiomyocytes revealed sustained expression. (B) Immunolocalisation of Orai1 and Stim1 in NCVM. Endogenous Orai1 (left panel) and Stim1 (right panel) are detected in NCVM and showed perinuclear enrichment. Cardiomyocytes are stained with actinin. Nuclear are stained with Sytox-Blue. (C) RT-PCR showing decreased expression of Orai1 mRNA in silenced cells compared to control cell (scrambled siRNA), 96 h after transfection ($n=6$ experiments, $^{*}p<0.01$). (D) Representative Western blots and densitometric analysis showing significant downregulation of Orai1 96 h after siRNA transfection ($n=6$ experiments, $^{*}p<0.01$). (E) RT-PCR showing decreased expression of Stim1 mRNA in silenced cells compared to control cell (scrambled siRNA), 96 h after transfection ($n=6$ experiments, $^{*}p<0.01$). (F) Representative Western blots and densitometric analysis showing significant downregulation of Stim1 96 h after siRNA transfection ($n=6$ experiments, $^{*}p<0.01$). (G) Representative records and quantitative analyses of SOCE in neonatal cardiac myocytes. Blue curve represents control cells treated with PE. Orai1-KD reduced SOCE (−75% decrease versus control group, Stim1-KD −62%, $p<0.05$, $n>30$ cells in each condition.). (H) Representative original traces and quantitative analyses of global Ca2+ transients in electrically stimulated control, Stim1 siRNA treated and Orai1 siRNA treated cardiomyocytes and representative original traces and quantitative analyses of SR Ca2+ load assessed by caffeine-induced cytosolic Ca2+ rise. Stim1 knockdown significantly decrease SR Ca2+ load ($p<0.05$, $n>40$ cells for each conditions).


A
Neonatal Cardiomyocytes
72kD
Stim1
32kd
Orai1
36kD
GAPDH
B
ORAI1
Actinin
Nuclei
STIM1
Actinin
Nuclei
C
Orai1 mRNA
P<0.05
n-fold change vs. HP/SCR normalized vs 18s
Control
Orai1KD
Stim1 KD
D
Orai1 Protein
P<0.05
n-fold change vs. HP/SCR normalized with GAPDH
Control
Orai1KD
Control
siRNA
32kD
Orai 1
36kD
GAPDH
E
Stim1 mRNA
P<0.05
n-fold change vs. HP/SCR normalized vs 18s
Control
stim1KD
Orai1 KD
F
Stim1 Protein
P<0.05
n-fold change vs. HP/SCR normalized with GAPDH
Control
Stim1 KD
Control
siRNA
72kD
Stim1
36kD
GAPDH
G
Control
Orai1KD
Stim1 KD
0 Ca2+ 2µM TG
1.8 mM Ca2+ 2µM TG
Intracelluler Ca2+ FURA-2 emission ratio (340/380)
60sec
SOCE FURA-2 emission ratio (340/380)
Control
Control + PE
Orai 1 Kd
Stim 1 Kd
H
Neonatal Cardiomyocytes
Control
Orai1 KD
Stim1 KD
Caffeine-induced FURA-2 emission ratio (340/380 nm)
2sec
20 mM caffeine
20 mM caffeine
20 mM caffeine
n.s.
Intracellular Ca2+ FURA-2 emission ratio (340/380)
Control
Orai 1 Kd
Stim 1 Kd
Caffein induced FURA 2 emmision ratio (SR-Content)
Control
Orai Kd
Stim Kd

cells due to SOCE, we first investigated expression levels both of Orai1 and Stim1 in isolated postnatal ventricular cardiomyocytes. Store operated Ca^{2+} Entry is one ubiquitous Ca^{2+} signalling pathway whereby depletion of intracellular Ca^{2+} stores activates Ca^{2+} channels in the plasma membrane. The Ca^{2+} entry via CRAC subsequently activates both CnA and CamKII pathways [8]. Interestingly, SOCE has been detected in embryonic, neonatal and adult cardiac myocytes [8,9] and we show here for the first time expression of Orai1 and Stim1 in cardiac myocytes and provide evidence that both proteins are required for SOCE in neonatal cardiomyocytes (Fig. 1(A)). Indirect immunofluorescent staining of cultured NRCMs (Fig. 1(B)) identifies a cytosolic and a primarily perinuclear pattern for Orai1 and Stim1, respectively. Control experiments with a blocking peptide lead to an absence of the Orai1 staining as shown in Supplemental Fig. 1(B).

Given the fact that IP3-receptors are predominantly expressed in the perinuclear region, this localisation points towards a role of Orai1 and Stim1 in the signalling between the plasma membrane and nucleus [10].

To investigate the role of Orai1 and Stim1 in NRCM silencing experiments with specific siRNAs were designed. Control studies with a fluorescent siRNA revealed an approximately 70% efficiency of the transfection under our conditions (Supplemental Fig. 1(A)).

An efficient decrease of Orai1 mRNA levels in cultured Orai1-siRNA treated NRCMs (Fig. 1(C)) and a subsequent approximately 5-fold decrease in Orai1 protein levels (Fig. 1(D)) after 96 h are sufficient to significantly attenuate SOCE activation in NRCMs due to ER/SR Ca^{2+} depletion (Fig. 1(G)). An equally efficient reduction in SOCE was seen after efficient mRNA knockdown of Stim1 resulting in an approximately 3-fold decrease of Stim1 protein in NRCMs (Fig. 1(E)). This means that Stim1 and Orai1are required for SOCE in neonatal cardiomyocytes. Importantly, SOCE could be significantly increased after treatment with phenylephrine (Fig. 1(G)). Analysis of Stim1 and Orai1mRNA abundance in Orai1- and Stim1 siRNA treated NRCMs, respectively, excluded significant reciprocal translational interference (Figs. 1(D) and (E)). No protein expression of Orai2, Orai3 and Stim2 were detectable in NRCM, however knockdown of Orai1 led to a significant increase of Orai2 mRNA level (Supplemental Fig. 2(A)), whereas knockdown of Stim1 led to significant increase of Stim2 mRNA level. In addition, analysis of transient receptor potential channel (TRPC) isoform 1, 3 and 6 mRNA expression levels, which convey LLC-dependent CnA activation in cardiomyocytes, showed unchanged abundance compared with controls (data not shown). The role of TRP-channels in SOCE is controversial, several lines of evidence argue against the notion that TRPC are store operated but rather are receptor-stimulated channels (ROC). However some studies could identify a role of TRPC1, TRPC3 and TRPC6 in the development of cardiac hypertrophy. Further studies are needed to clarify this issue [11–13].

Further analysis of Ca^{2+} fluxes involved in the regulation of NRCM contractility revealed that Orai1 siRNA treated NRCMs exhibit both unchanged cytosolic Ca^{2+} transients (Fig. 1(H)) and caffeine-releasable Ca^{2+} from the SR (Fig. 1(H)) compared to controls. In contrast, siRNA-mediated knockdown of the Ca^{2+} sensor Stim1 impacted both cytosolic and SR Ca^{2+} handling as reflected by significantly lower diastolic Ca^{2+}levels (data not shown) and a decline in SR Ca^{2+} content (Fig. 1(H)). Therefore Stim1 might have a role in maintaining the available Ca^{2+} in the SR by sensing the luminal Ca^{2+} concentration. Given the fact that increased SR Ca^{2+} leak in heart failure leads to a decreased SR Ca^{2+} content, it is tempting to speculate that Stim1 senses the decreased Ca^{2+} concentration and signals to Orai1 channels to maintain the SR Ca^{2+} store, leading to the activation of Ca^{2+} dependent growth via activation of CnA or CamK and contributes to pathological hypertrophy. Also the frequency of spontaneous Ca^{2+} transients were significantly altered and decreased in Orai1 and Stim1-KD cardiomyocytes (Supplemental Fig. 2(B)).

In light of previously reported defects in skeletal muscle growth in mice with genetically manipulated Orai1 and Stim1 protein levels [14], we determined the impact of decreased Orai1 and Stim1 protein levels on normal (eutrophic) postnatal growth. Assessment of the cell surface area revealed a significant reduction in cell size of α-actinin immunostained NRCMs with Orai1-KD versus control (Figs. 2(A) and (B)). Subsequent analysis of Ca^{2+} regulated pathways showed a significant reduction in the activity of both the CnA and calmodulin kinase II cascade. This is reflected by significantly decreased expression of the CnA target gene mcip.1.4 (Fig. 2(C)) along with a decline of *bnp* and *anp* mRNA levels in Orai1 siRNA treated NRCMs (Fig. 2(D)) versus controls.

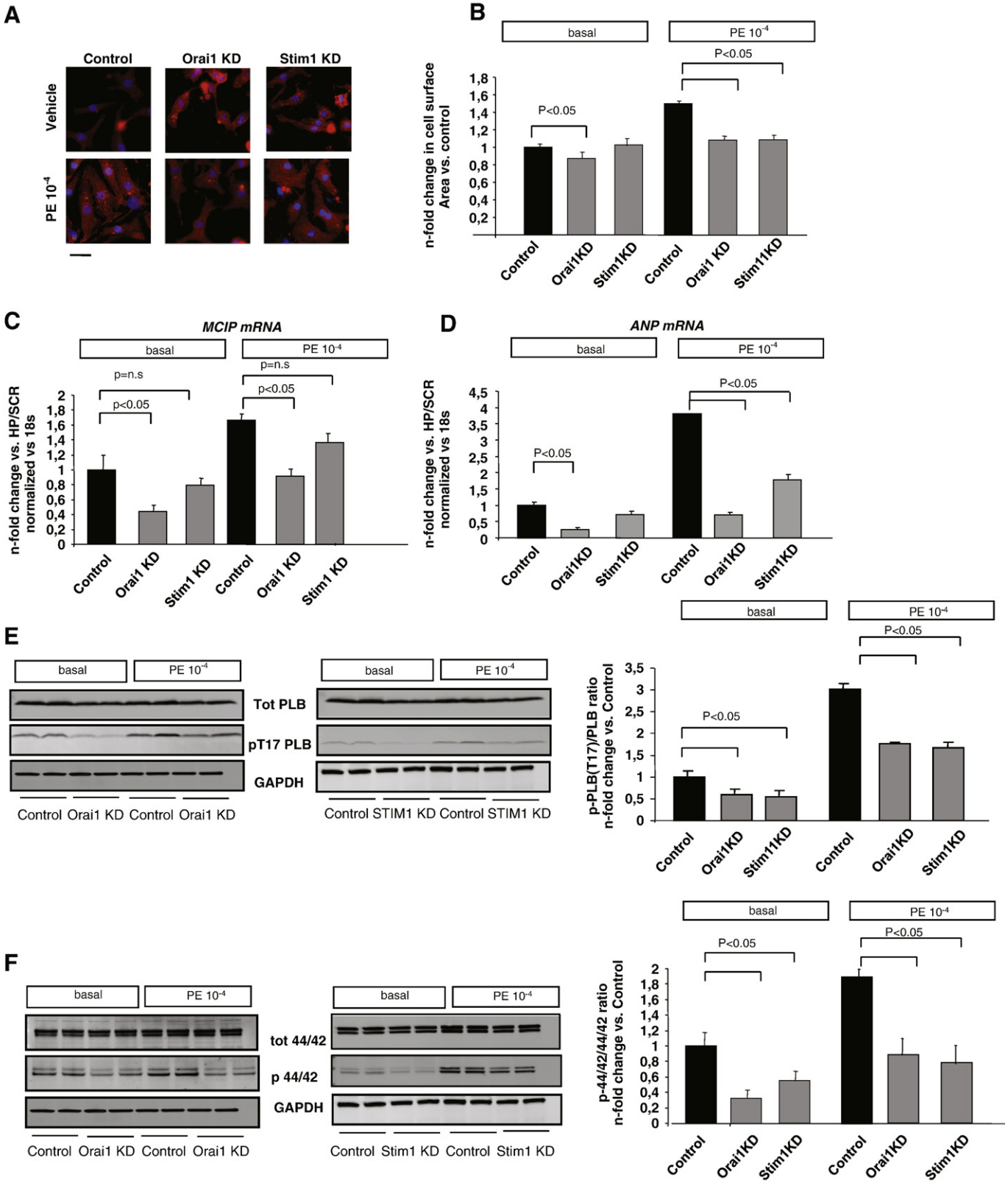
Furthermore, we found significantly diminished phosphorylation of the phospholamban threonine 17 site (Fig. 2(E)) representing reduced basal CamKII activity in NRCMs with diminished Orai1 expression. Interestingly, extracellular Ca^{2+} influx has recently been linked to activation of the prohypertrophic kinase ERK1/2 that is involved in NRCM growth regulation. We therefore determined levels of phosphorylated ERK1/2 and found a significant reduction in ERK1/2 activity (Fig. 2(F)). In contrast, Stim1-KD neither influenced NRCM size (Figs. 2(A) and (B)) nor CaN activity (Fig. 2(C)) under basal culture conditions but diminished CamKII (Fig. 2(E)) and ERK1/2 activity (Fig. 2(F)), indicating that either activation of different Ca^{2+} channels, i.e. receptor activated TRPC channels, compensate for the knockdown or the remaining Stim1 proteins are sufficient for interaction with Orai1. It is important to note that several studies indicate that TRPC channels are gated by STIM1 and that TRPC have two operating modes: Stim1-dependent and -independent and the extent of interaction with Stim1 (level of endogenous Stim1) might determine if they function as SOCs or as receptor-stimulated channel in a given cell type [15]. Clearly further studies are required to examine the relationship between TRPC, Orai1 and Stim1.

Cardiac hypertrophy is associated with arrhythmias, myocyte death and congestive heart failure and is an independent predictor of cardiovascular morbidity and mortality [16]. Therefore, we sought to determine the role of Orai1 and Stim1 in G*q*-protein mediated hypertrophic growth of NRCMs. As expected, control NRCMs responded with an approximately 50% increase in cell size (Fig. 2(A)), enhanced *anp* and *bnp* expression (Fig. 2(D)), and augmented CnA (Fig. 2(C)), CamKII (Fig. 2(E)) and ERK1/2 (Fig. 2(F)) activity. Note that decreasing either Orai1 or Stim1 protein alone is sufficient to exert a profound anti-hypertrophic effect and completely abrogated the PE-mediated increase in NRCM cell size (Fig. 2(A)) and enhanced expression of natriuretic factors. In line with these results, Orai1-KD prevented PE-mediated activation of CaN, CamKII and ERK1/2 versus controls. Similar results were obtained in Stim1 siRNA treated NRCMs with the exception that Stim1-KD did not block PE-mediated CnA activation. In addition we analysed the nuclear localisation of NFAT

Fig. 2. Orai1 and Stim1 are required for hypertrophic growth. (A) Representative images of control (scramble siRNA) and siRNA treated cardiac myocytes stained with an alpha-actinin antibody. DAPI is used as stain for the nuclei, scale bars 10 m. (B) Statistical analysis of the cell surface. While stimulation with PE for 48 h led to a 1.50 fold increase in surface area in control cells, STIM1 or Orai1 knockdown cells resist the hypertrophic stimulus ($n=4$ $^*p<0.05$). (C) Relative MCIP1.4 mRNA expression levels at baseline and PE-treated neonatal rat ventricular cardiomyocytes. A significant induction of MCIP1.4 (1.6 fold) was observed in PE stimulated control cells, whereas Orai1-siRNA treated neonatal rat ventricular cardiomyocytes have reduced MCIP1.4 mRNA levels at baseline and after treatment with PE. (D) Stim1or Orai1 knockdown prevents induction of BNP after PE treatment ($n=4$ $^*p<0.05$). (E) Representative pictures and densitometric analysis of Western blots of GAPDH, total PLB and phospho (T17) PLB. Knockdown of Orai1 (left panel) and Stim1 (right panel) decreases levels of phosphorylated PLB (t17-PLB) at baseline and prevents increase in phosphorylation after PE treatment for 48 h ($p<0.05$, $n=3$). Total PLB is unchanged. GAPDH was used for standardizations. (F) Representative pictures and densitometric analysis of Western blots of GAPDH, total p44/42 and phospho p44/42. Knockdown of Orai1 (left panel) and Stim1 (right panel) decreases levels of phosphorylated p44/42 at baseline and prevents increase in phosphorylation after PE treatment for 48 h ($p<0.05$, $n=3$). Total p44/42 is unchanged. GAPDH was used for standardization.

using an EGFP-NFAT adenovirus. Phenylephrines lead in accordance with the mcip.1.4 mRNA levels to an increased nuclear localisation of EGFP-NFAT, whereas Orai1-KD prevented the nuclear localisation (Supplemental Fig. 2(C)).

Thus targeting of the Stim1/Orai1 pathway could represent a good strategy to prevent pathological hypertrophy. The role of Stimrt1 and Orai1 in intact hearts remains to be determined. Mice deficient for Stim1 or Orai1 die shortly after birth [14,17], however so far no



mechanisms for the postnatal death have been reported. No cardiac phenotype has been described in patients with mutations in Orai1 or Stim1 proteins but it is known that almost all of the patients develop myopathies. Conversely, it is well known from other myopathies (i.e. Duchenne) that many of patients with myopathies develop also a cardiomyopathy with time. It will be therefore be interesting to investigate the role of Orai1 and Stim1 in vivo and in human hearts.

In summary, we showed that Orai1 and Stim1 together play a key role in cardiomyocyte SOCE, regulating both normal and hypertrophic postnatal cardiac growth in vitro. Moreover Stim1 seems also to play a role in sensing the SR Ca^{2+}. Mechanistically, the effects of Orai1 knockdown are likely mediated via reduced CnA and CamKII activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2010.01.020.

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