HHS Public Access

Author manuscript

J Mol Cell Cardiol. Author manuscript; available in PMC 2017 July 15.

Published in final edited form as:

J Mol Cell Cardiol. 2010 June ; 48(6): 1329–1334. doi:10.1016/j.yjmcc.2010.01.020.

Orai1 and Stim1 Regulates 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) signaling pathway although diminishing both Orai1 and Stim1 protein similarly attenuated calmodulin kinase II (CamKII) and

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Disclosures: none declared

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 signaling 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.

Keywords

Stim1; Orai1; hypertrophy; cardiac; calcium

Introduction

Cardiac myocytes express a large number of Ca^{2+} signaling 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 are 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 77kD 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 32kD 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+}) channels channel pore-forming subunit protein. Ca^{2+} release by IP-3 Receptors causes Stim1 oligomerization and relocalization into ER-plasma membrane junctions. This subsequently activates Orai1 in the adjacent plasma membrane and cause Ca^{2+} entry into the cytosol via CRAC that mediate Ca^{2+} signals required for activation of CamK and CnA.

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.

Material and Methods

Isolation and primary culture of neonatal rat ventricular cardiomyocytes

Ventricular cardiomyocytes from 1–2 day old rat neonatal hearts (NRCMs) were prepared by trypsin digestion as described previously [6]. After 24h the medium was replaced by a 0.5% serum-containing DMEM and NRCM were transfected with different siRNAs. 48h after transfection cells were stimulated with phenylephrine for up to 48h. 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 siRNA's were transfected on the same day with a EGFP-NFAT adenovirus and the cellular localisation of EGFP-NFAT were analysed 48 h after transfection and 24 h after treatment with Phenylephrine.

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 (5nM) 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

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 hours cells were fixed, permeabilized and labeled with antibodied specific to Stim1, Orai1 and sarcomeric actinin followed by the corresponding Cy3-conjugated, Cy5-conjugated or FITC-conjugated secondary anibodies (Jackson ImmunoResearch Lab) (1/100). Confocal images (CLSM) were obtained using a 63x water objective on a Leica TCS SP laser scanning confocal microscope. Digitized confocal images were processed by Adobe Photoshop.

Intracellular Ca^{2+}-measurements

Intracellular Ca^{2+}-transients of Fura 2-AM loaded (2 μmol/L) NRCM were obtained 96 hours 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 analyzed 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 (10mM) in conjunction with the SERCA inhibitor thapsigargin (1μM) to prevent store refilling and archieve maximal store depletion. Verapamil was added to prevent L-type Ca^{2+} entry (10μM) to the solutions. Relative changes in intracellular Ca^{2+} were monitored following reperfusion of

2mM 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 488nm and emission detection >515nm) were obtained on a Leica TCS SP laser scanning confocal microscope with a 20x water immersion objective. Time-lapsed (xy, 200ms/frame) were obtained.

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

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.

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 1μg 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.

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.

Results and Discussion

Since Orai1 and Stim1 orchestrate Ca^{2+}-dependent regulation of skeletal muscle growth and key biological functions in non-cardiac cells due to SOCE, we first investigated expression levels both of Orai1 and Stim1 in isolated postnatal ventricular cardiomyoctes. Store operated Ca^{2+} Entry is one ubiquitous Ca^{2+} signaling 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 1A).

Indirect immunofluorescent staining of cultured NRCMs (Figure 1B) 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 the the Supplement Figure 1B.

Given the fact that IP3-receptors are predominantly expressed in the perinuclear region, this localization 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 a approximately 70% efficiency of the transfection under our conditions (Supplemental Fig 1A).

An efficient decrease of Orai1 mRNA levels in cultured Orai1-siRNA treated NRCMs (Fig 1C) and a subsequent approximately 5-fold decrease in Orai1 protein levels (Figure 1D) after 96h is sufficient to significantly attenuate SOCE activation in NRCMs due to ER/SR Ca^{2+} depletion (Figure 1G). 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 (Figure 1E). This means that Stim1 and Orai1are required for SOCE in neonatal cardiomyocytes. Importantly, SOCE could be significantly increased after treatment with phenylephrine (Figure 1G). Analysis of Stim1 and Orai1mRNA abundance in Orai1- and Stim1-siRNA treated NRCMs, respectively, excluded significant reciprocal translational interference (Figure 1D 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 Figure 2A), 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 (Figure 1H) and caffeine-releasable Ca^{2+} from the SR (Figure 1H) 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 (Figure 1H). 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 (Figure 2A 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 (Figure 2C) along with a decline of bnp and anp mRNA levels in Orai siRNA treated NRCMs (Figure 2D) versus controls.

Furthermore, we found significantly diminished phosphorylation of the phospholamban threonin 17 site (Figure 2E) 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 (Figure 2F). In contrast, Stim1-KD neither influenced NRCM size (Figure 2A and B) nor CaN activity (Figure 2C) under basal culture conditions but diminished CaMKII (Figure 2E) and ERK1/2 activity (Figure 2F), 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 (Figure 2A), enhanced anp and bnp expression (Figure 2D), and augmented CnA (Figure 2C), CaMKII (Figure 2E) and ERK1/2 (Figure 2F) 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 (Figure 2A) 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 analyzed the nuclear localiszation of NFAT 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 Figure 2C).

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by grants of the National Institute of Health (RO1 HL92130 and RO1HL92130-02S1 to P. Most), Deutsche Forschungsgemeinschaft (562/1-1 to P.Most and VO1659/1-1 to M.Voelkers) and Bundesministerium fuer Bildung und Forschung (01GU0572 to P.Most).

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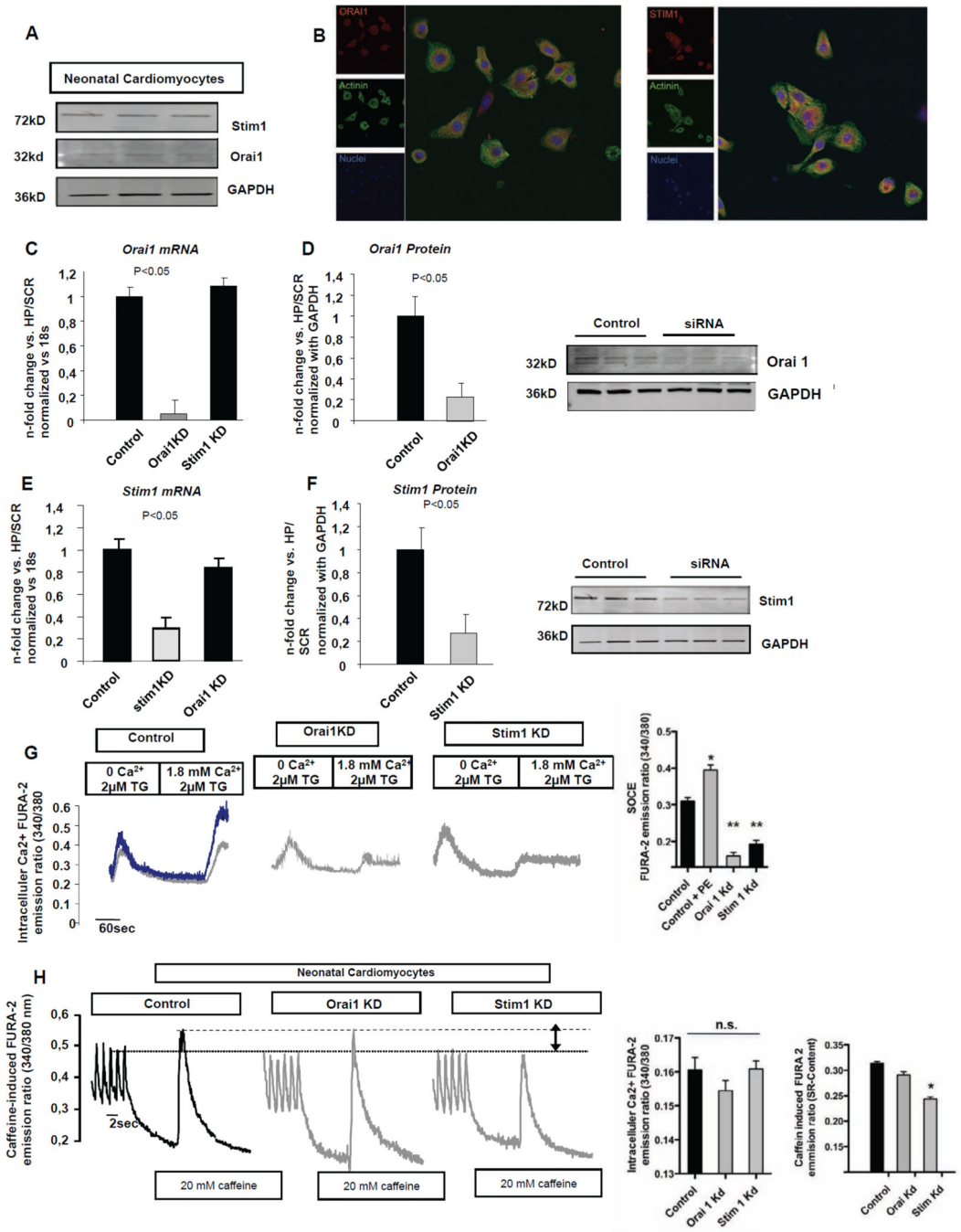

Figure 1.

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**, Immunolocalization 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), 96h after transfection (n=6 experiments, *

p<0.01), **D**, Representative Western Blots and densitometric analysis showing significant downregulation of Orai1 after 96h 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), 96h after transfection (n=6 experiments, * p<0.01). **F**, Representative Western Blots and densitometric analysis showing significant downregulation of Stim1 after 96h 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 vs control group, Stim1 KD −62%, p<0.05, n>30 cells in each condition.). **H** Representative original traces and quanttitative analyses of global Ca^{2+} transients in electrically stimulated control, Stim1 siRNA treated and Orai1 siiRNA treated cardiomyocytes and representative original traces and quanttitative analyses of SR Ca^{2+} load assessed by caffeine-induced cytosolic Ca^{2+} rise. Stim1 knockdown significantly decrease SR Ca2+ load (p<0.05, n>40 cells for each conditions)

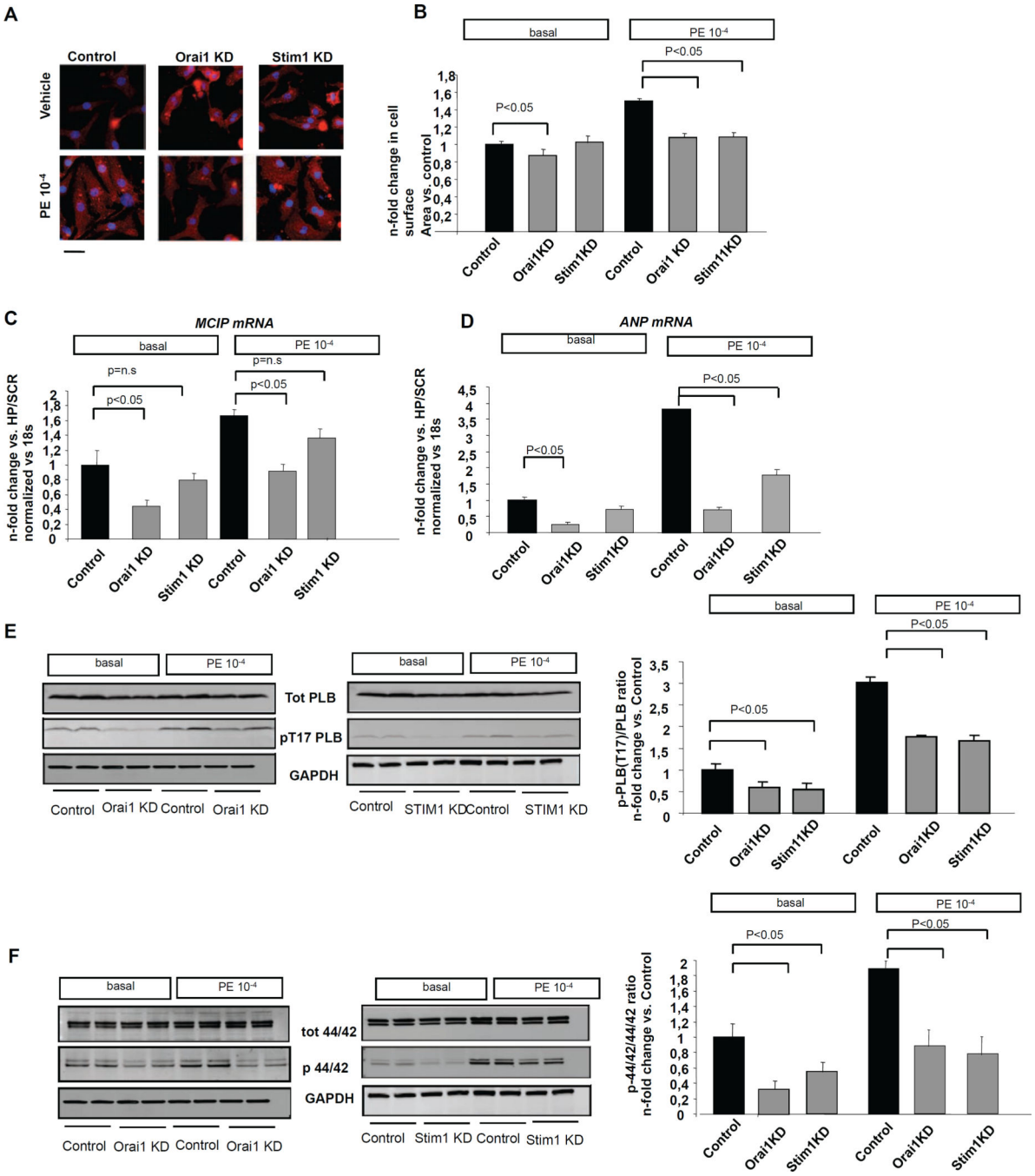

Figure 2.

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 48h 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 and 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 prevent induction of BNP after PE treatment (n=4* $p<0.05$). **E** Representative Pictures and densidometric analysis of Western Blots of GAPDH, total PLB and phospho (T17) PLB. Knockdown of Orai1 (left panel) and Stim1 (right panel) decrease levels of phosphorylated PLB (t17-PLB) at baseline und prevents increase in phosphorylation after PE treatment for 48h ($p < 0.05$, $n = 3$). Total PLB are unchanged. GAPDH was used for standardizations. **F** Representative Pictures and densidometric analysis of Western Blots of GAPDH, total p44/42 and phospho p44/42. Knockdown of Orai1 (left panel) and Stim1 (right panel) decrease levels of phosphorylated p44/42 at baseline und prevents increase in phosphorylation after PE treatment for 48h ($p < 0.05$, $n = 3$). Total p44/42 are unchanged. GAPDH was used for standardization