Abstract. RhoE/Rnd3 is an atypical member of the Rho super-family of proteins. However, the global biological function profile of this protein remains unsolved. In the present study, a RhoE-knockout H9C2 cardiomyocyte cell line was established using CRISPR/Cas9 technology, following which differentially expressed genes (DEGs) between the knockout and wild-type cell lines were screened using whole genome expression gene chips. A total of 829 DEGs, including 417 upregulated and 412 downregulated, were identified using the threshold of fold changes ≥1.2 and P<0.05. Using the ingenuity pathways analysis system with a threshold of -Log (P-value)>2, 67 canonical pathways were found to be enriched. Many of the detected signaling pathways, including that of oncostatin M signaling, were found to be associated with the inflammatory response. Subsequent disease and function analysis indicated that apart from cardiovascular disease and development function, RhoE may also be involved in other diseases and function, including organismal survival, cancer, organismal injury and abnormalities, cell-to-cell signaling and interaction, and molecular transport. In addition, 885 upstream regulators were enriched, including 59 molecules that were predicated to be strongly activated (Z-score >2) and 60 molecules that were predicated to be significantly inhibited (Z-scores <2). In particular, 33 regulatory effects and 25 networks were revealed to be associated with the DEGs. Among them, the most significant regulatory effects were ‘adhesion of endothelial cells’ and ‘recruitment of myeloid cells’ and the top network was ‘neurological disease’, ‘hereditary disorder, organismal injury and abnormalities’. In conclusion, the present study successfully edited the RhoE gene in H9C2 cells using CRISPR/cas9 technology and subsequently analyzed the enriched DEGs along with their associated canonical signaling pathways, diseases and functions classification, upstream regulatory molecules, regulatory effects and interaction networks. The results of the present study should facilitate the discovery of the global biological and functional properties of RhoE and provide new insights into role of RhoE in human diseases, especially those in the cardiovascular system.

Introduction

RhoE, also known as rho family GTPase (Rnd) 3 (Rnd3) or small GTP-binding protein Rho8, encodes a protein that belongs to a member of the small GTPase protein superfamily (1). Unlike the traditional Rnd family of proteins, RhoE cannot switch from the GTP-bound to GDP-bound forms to mediate its activity (1). Since it was first identified in 1996, many physiological and pathophysiological functions of RhoE have been uncovered (2-5). Early studies suggested that RhoE was associated with RhoA/Rho-associated coiled-coil kinase signaling in regulating actin cytoskeleton organization (6,7). Studies performed over the past decade using RhoE gene-edited cells and animal models have revealed additional RhoE functions (8-12), which highlighted the complex role of RhoE in mammalian diseases. A more comprehensive understanding of the function and interactions mediated by RhoE, in addition to the signaling pathways associated with this protein,
may provide novel insights into the role of RhoE in human health and diseases.

Cardiovascular disease presents a significant threat to human health. RhoE has been previously reported to promote endothelial barrier recovery during inflammatory challenge (13) and alleviate vascular injury caused by insulin resistance (14). The successful establishment of RhoE-knockout and RhoE-overexpressing mouse models has allowed the discovery of the relationship between RhoE and cardiovascular diseases. Yue et al (15) previously generated the RhoE<sup>−/−</sup> haplinsufficient mouse model, which is predisposed to transverse aortic constriction stress and develop apopotic cardiomyopathy with heart failure. Additionally, the same research group also found impaired angiogenesis in this animal model through destabilization of the hypoxia inducible factor 1α-vascular endothelial growth factor-A signaling pathway (8). Subsequently, it was revealed further that RhoE-null mice were embryonically lethal with cardiac arrhythmias due to the aberrant activation of the β-adrenergic signaling pathway (10). By applying global RhoE-knockout, cardiomyocyte-specific RhoE heterozygous and cardiomyocyte-specific RhoE overexpressing mice, it was found recently that RhoE regulated myocardial zygous and cardiomyocyte-specific function (9). The successful establishment of RhoE-knockout and cardiomyocyte-specific overexpression mouse models has allowed the discovery of the relationship between RhoE and cardiovascular diseases. Furthermore, it was revealed further that RhoE-null mice were embryonically lethal with cardiac arrhythmias due to the aberrant activation of the β-adrenergic receptor-protein kinase A signaling pathway (10). By applying global RhoE-knockout, cardiomyocyte-specific RhoE heterozygous and cardiomyocyte-specific RhoE overexpressing mice, it was found recently that RhoE regulated myocardial infarction-induced inflammation and promoted cardiac recovery from injury by mediating NF-κB signaling (12). Taken together, these results aforementioned suggest that the targeted manipulation of RhoE can be a potential method of therapeutic intervention for major cardiovascular diseases.

To elucidate the role of RhoE in cardiovascular diseases, a RhoE-knockout H9C2 cardiomyocyte cell line was produced using CRISPR/Cas9 technology. Whole genome expression gene chip technology was then used to detect differentially expressed genes (DEGs) between the RhoE-null and wild-type H9C2 cell models. By performing ingenuity pathway analysis (IPA) using the IPA software (version 42012434; Ingenuity Systems; Qiagen China Co., Ltd.), the global profiles of signaling pathways, functions and interactions associated with RhoE were obtained. Data obtained from the present study provide a general overview of the role and targeted intervention of RhoE during pathological conditions in the cardiovascular system.

**Materials and methods**

**Cell culture.** H9C2 cardiomyocytes and 293T cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The cells were maintained in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (Life Technologies; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere under 5% CO<sub>2</sub>. The medium was changed every 3 days. Cells were routinely tested for contamination with mycoplasma or chlamydia before being used in subsequent experiments.

RhoE-knockout in H9C2 cardiomyocytes using CRISPR/Cas9 technology. Obtaining the RhoE<sup>−/−</sup> mice was difficult due to high embryonic lethality after E11.5 (2). Therefore, CRISPR/Cas9 technology was used to edit the RhoE gene in H9C2 cardiomyocytes. The GV392 plasmid (also named LV-hspCas9-P2A-puro; Shanghai GeneChem Co., Ltd.) was used (16). Three single guide RNAs (sgRNAs) specific to rat RhoE and one scramble sgRNA were designed using the Cas-Designer online web tool (http://www.rgenome.net/cas-designer/) (17), where their associated oligonucleotide sequences were synthesized by Shanghai GeneChem Co., Ltd. The sequences of the sgRNA oligonucleotides are listed in Table S1. The oligonucleotides were then ligated into the linearized lentiviral vector GV392. Following verification of the plasmids by Sanger sequencing on the Shanghai GeneChem Co., Ltd. platform, lentiviral particles were produced in 293T cells by co-transfecting them with the plasmid Helper 1.0 and Helper 2.0 plasmids (Shanghai GeneChem Co., Ltd.) using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) for 48 h before collection. The masses of the LV-hspCas9-P2A-puro, Helper 1.0 and Helper 2.0 plasmids used were 20, 15 and 10 µg per transfection reaction in 10-cm dishes. The physical structures of the plasmids and the sequences of the sgRNA are shown in Fig. S1. To identify the functionality of the sgRNA, H9C2 cells were infected with the lentiviral particles at multiplicity of infection of 100. At 48 h post-infection 2.0 µg/ml puromycin was added to stress the cells continuously for 5 days. Genomic DNA was then extracted using the TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd.), which was subjected to semi-quantitative PCR analysis to measure RhoE expression. For the PCR analysis, 2X Taq Plus Master Mix (Vazyme Biotech Co., Ltd.) was used and thermocycling conditions were: Initial denaturation at 95°C for 90 sec, followed by 35 cycles of 95°C for 20 sec, 55°C for 20 sec and 72°C for 50 sec. Following a final cycle of 98°C for 3 min, the products were cooled at room temperature to below 40°C. Knockout and Mutation Detection kit (cat. no. MB001-100420mx; Shanghai Genesci Medical Technology Co., Ltd.) was used to verify RhoE-knockout in H9C2 cells. PCR products were treated with Detecase with the suitable buffer under 45°C for 20 min. Subsequently, the products were subjected to 2.0% agarose gel electrophoresis containing SyBR™ Green I Nucleic Acid Gel Stain solution (cat. no. S7563; Thermo Fisher Scientific, Inc.) and visualized using Tanon 4200 gel imaging system (version 1.0, Tanon Science and Technology Co., Ltd.). Since the cells in the RhoE-knockout group is of mixed clone, mutation of the same target site among the cells is different. After annealing, the PCR product can form a mismatch site and be recognized by the mismatch enzyme Detecase, which can detect the activity of sgRNA according to the cleaved bands. The PCR product of the Cdc42 gene (Shanghai GeneChem Co., Ltd.) served as the positive control. The primers for PCR and respective product sizes before and after crusing nucleases digestion are provided in Table SII. Finally, the functional lentiviral particles were used to infect H9C2 cardiomyocytes at multiplicity of infection of 100. Puromycin (2.0 µg/ml) was added to stress the cells continuously for 7 days, before western blotting was performed to measure the RhoE protein expression and total RNA was subjected to whole genome expression profile chip analysis.

**Microarray experiments using the GeneChem platform.** The microarray experiments were performed by Shanghai GeneChem Co., Ltd. Briefly, total RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher
Western blotting. The procedure was performed as previously described (23,25). In brief, cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology), where the consequent protein samples were quantified using the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). In total, 30 µg proteins from each sample were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After washed and incubated with 5% skimmed milk in TBS-0.05% Tween-20 at 37°C for 2 h, membranes were probed with primary antibodies at 4°C overnight. After two washes with TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 37°C for 1 h. The following antibodies were used: RhoE (1:200; cat. no. 66228-1-Ig; ProteinTech Group, Inc.), osteoprotegerin (TNFRSF11B; 1:500; cat. no. ab73400; Abcam), Casitas B-lineage lymphoma (CBL; 1:500; cat. no. ab119954; Abcam), structural maintenance of chromosomes 1A (SMC1A; 1:500; cat. no. ab133643; Abcam), cofilin-1 (CFL1; 1:500; cat. no. 5175; Cell Signaling Technology, Inc.), eukaryotic translation initiation factor 4e binding protein 1 (EIF4EBP1; 1:1,000; cat. no. 9644; Cell Signaling Technology, Inc.), SHC adaptor protein 1 (SHC1; 1:500; cat. no. 2432; Cell Signaling Technology, Inc.), cyclin D2 (CCND2; 1:1,000; cat. no. 3741; Cell Signaling Technology, Inc.), cyclin B1 (CCNB1; 1:1,000; cat. no. 4138; Cell Signaling Technology, Inc.), eukaryotic translation initiation factor 2 subunit (EIF2S2; 1:500; cat. no. 5324; Cell Signaling Technology, Inc.), STAT3 (1:500, cat. no. 9139, Cell Signaling Technology, Inc.), GAPDH (ΔΔCq method (24) was used to quantify the expression levels of target genes, with GAPDH as the internal control.

**Quantitative PCR (qPCR).** To verify the chip results, 20 genes of interest that were associated with the enriched canonical pathways were selected for qPCR analysis. cDNA was generated from 1.0 µg RNA using a RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). In total, 20 µl reactive mixture and the SYBR® Premix Ex Taq™ II kit (Takara Bio, Inc.) were used for the qPCR, as described previously (22,23). The sequences of the primers are provided in Table SIII. The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, 60°C for 10 sec and 72°C for 20 sec. The 2−ΔΔCq method (24) was used to quantify the expression levels of target genes, with GAPDH as the internal control.

**Statistical analysis.** The IPA system (version 42012434) was used to perform bioinformatics analysis. Pearson's correlation was used to compare the correlation of different groups of microarray hybridization signals. The right-tailed Fisher's exact test and unpaired t-test were used for analysis and validation of DEGs, P<0.05 was considered to indicate a statistically significant difference. For the IPA analysis, the P-value, -log (P-value), P-value of overlap, Z-score and consistency scores were used, which were mentioned above. For producing of the heatmaps and analysis of qPCR results, GraphPad Prism (version 8.0, GraphPad Software, Inc.) was used.
Results

Effective sgRNA designed against rat RhoE. Three sgRNAs that target the rat RhoE DNA sequence were designed and packaged into lentiviruses using 293T cells. PCR was subsequently performed using the genome DNA samples derived from the virus-infected H9C2 cells. According to the cruiser enzyme digestion method, the heterologous double-stranded DNA with a mutation site can be efficiently cleaved from the 3′ end of the mutation site by the nuclease, thereby generating fragments of different sizes. In the present study, after Cruiser digestion, the 838 bp product of NC-1 and KO-2 (sgRNA-2) could not generate any bands at 303 and 530 bp, whilst the 738 bp product of NC-2 and KO-3 (sgRNA-3) could not generate any bands at 247 and 491 bp. However, KO-1 (sgRNA-1) labeled, as indicated by the white asterisk, indicated that the lentivirus of interest had the correct sgRNA sequence, as it yielded a band at 303 and another at 530 bp after cruiser digestion (Fig. 1A). This suggests that in conjunction with the Cas9 protein, sgRNA-1 functioned well in H9C2 cells, since it induced the correct mutation in the PCR products of the RhoE DNA exon sequence. In cells transfected with sgRNA-1, the western blotting results showed that RhoE protein expression was significantly suppressed, even in mixed cloned H9C2 cell pools consisting of cells with different RhoE DNA mutation efficiencies (Fig. 1B), indicating a depletion of RhoE protein post editing. The heat maps representing RhoE mRNA expression in the control and knockout cells and the chip results further verified that the RhoE mRNA levels in the knockout group were significantly lower compared with those in cells transfected with scrambled sgRNA (NC; Fig. 1C and D).

RhoE-knockout in H9C2 cells leads to significant changes in gene expression. Rigorous quality control of the RNA samples and chip data was conducted before the data were analyzed (Figs. S2 and S3). The RhoE-knockout H9C2 cells had a total of 829 DEGs compared with those in the wild-type control cells (FC ≥1.2 and P<0.05), in which 417 were found to be upregulated and 412 were downregulated (Fig. 2A and B). The top five upregulated genes were LOC100912383, TNN, CIU, IL33 and ATP7A, whilst the top five downregulated genes were WASI, HIST1H1D, ID11, SCD1 and TBLIX (Fig. 2B). The heat maps representing the top 20 upregulated and top 20 downregulated genes are shown in Fig. 2C and D.

Enriched canonical pathways detected using IPA. The complete list of enriched canonical pathways is included in Table SIV. A total of 67 enriched canonical pathways were identified by applying the -log (P-value)>2 threshold, top 25 of the 67 representative pathways ranked according to their -log (P-value) that were found to associate tightly with the cardiovascular system are shown in Fig. 3, along with the number of enriched genes that function within each of these signaling pathways. The ‘superpathway of cholesterol biosynthesis’ was the highest ranking signaling pathway with a -log (P-value) of 17.5. Taking Z-score >2 as the threshold of significant activation, it was subsequently found that ‘androgen signaling’ (Z-score=2.236), ‘interferon signaling’ (Z-score=2.236), ‘nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated oxidative stress response’ (Z-score=2.236), ‘Gβγ signaling’ (Z-score=2.236), ‘oncostatin M signaling’ (Z-score=2.121) and ‘thrombopoietin signaling’ (Z-score=2.121) were significantly activated (Table SIV). Although none of the detected signaling pathways had a Z-score <-2, some of the enriched signaling pathways had Z-scores <1 (Table SIV), including the ‘osteoarthritis pathway’ (Z-score=−1.508), ‘Wnt/β-catenin signaling’ (Z-score=−1.414), ‘TGFB-β signaling’ (Z-score=−1.414), ‘regulation of actin-based motility by Rho’ (Z-score=−1.414), ‘NF-kB signaling’ (Z-score=−1.134), ‘cell division cycle 42 (Cdc42) signaling’ (Z-score=−1.134), ‘glioma invasiveness signaling’ and ‘signaling by Rho family GTPases’ (Z-score=−1.069).

Upstream regulator analysis. Upstream regulators, including transcription factors, cytokines, small RNAs, receptors, kinases, chemical molecules and pharmacological agents can all regulate gene expression. By applying the P-value of overlap <0.05 threshold, a total of 885 upstream regulators were enriched (Table SV). Among them, 59 had activation Z-scores >2 and 60 had activation Z-scores <-2. The top 15 activated and top 15 inhibited upstream regulators are listed in Table 1. Cholesterol was found to be the most powerful activator (Z-score=4.423; P-value of overlap=5.86x10⁻12). In addition, the following 32 target molecules in the dataset were enriched: ABCA7, ACS2, BSG, CD14, CXCL2, CYP7A1, DHCR7, FADS1, FASN, FDF1T, FDPS, HMGCR, ID1, INHA, LDLR, LGALS3, MMP14, MCMO1, NSDHL, PMVK, PSTAT1, RPL36A1, SCD5, SCD, SLCA13, SLC7A5, SQCS, SLE, TIMP3, TM7SF2, UXT, and VACM1. Sterol regulatory element-binding protein cleavage-activating protein (SCAP) was revealed to be the most powerful inhibitor (Z-score=−4.617; P-value of overlap=2.12x10⁻18) with the following 22 target molecules in dataset also enriched: ACS2, CYP51A1, DHCR7, ELOVL6, FASN, FDF1T, FDPS, HMGCR, ID1, INSI1, LDLR, LSS, MCMO1, MVD, MVK, NSDHL, PMVK, SCD5, SCD, SLE, TM7SF2, and TMEM97.

Disease and function analysis. By applying the log(P-value) >4 threshold on the IPA system, the role of RhoE in diseases and in cellular function was next determined. The complete disease and function classifications are provided in Table SVI. The top categories ranked in accordance with their -log(P-value) are shown in Fig. 4. A histogram containing some representative classifications of disease and function is shown in Fig. 4A and the representative heatmap is shown in Fig. 4B. RhoE was found to serve important roles in a number of diseases and cellular functions, including ‘organismal survival’ [-log(P-value)=23.567; Z-score=−2.362], ‘cancer, organismal injury and abnormalities’ [-log(P-value)=12.551; Z-score=−3.043], ‘cell death and survival’ [-log(P-value)=5.577; Z-score=−2.068], ‘cell-to-cell signaling and interaction’ [-log(P-value)=10.79; Z-score=−2.54], ‘cellular function and maintenance’ [-log(P-value)=6.807; Z-score=−2.07] and ‘molecular transport’ [-log(P-value)=4.137; Z-score=−2.832]. With the focus on cardiovascular disease and development function, RhoE was demonstrated to serve a role in 36 aspects, including ‘disorder of blood pressure’ [-log(P-value)=5.019; Z-score=−1.000], ‘hypertension’ [-log(P-value)=4.724; Z-score=0.056], ‘cell death of cardiomyocytes’ [-log(P-value)=4.119; Z-score=0.956], ‘abnormality of heart ventricle’ [-log(P-value)=7.080; Z-score=0.923], ‘binding of
endothelial cells' [-log(\(P\)-value)=5.678, Z-score=2.729], ‘adhesion of endothelial cells’ [-log(\(P\)-value)=4.830, Z-score=2.444], ‘angiogenesis’ [-log(\(P\)-value)=8.197, Z-score=0.024] and ‘vasculogenesis’ [-log(\(P\)-value)=7.381, Z-score=2.444], and ‘cardiovascular system development and function’ [-log(\(P\)-value)=4.830, Z-score=2.729], ‘adhesion of endothelial cells’ [-log(\(P\)-value)=5.678, Z-score=2.729], ‘neurological disease, hereditary disorder, organismal injury and abnormalities’, involving 35 molecules (Table SVIII). Among them, the highest ranked regulatory function (27). In total, 33 types of regulatory effects were found (Table SVII). Among them, the highest ranked regulatory effect with a consistency score of 8.043 strongly suggests that the RhoE-knockout-induced DEGs CD28, CHUK, IFNAR1, IKBKG, INSR, and RIPK2 may be involved in the adhesion of endothelial cells and recruitment of myeloid cells, mainly through mediating their targets, including AXL, CXCL12, HSPA1A/HSPA1B, CYP1B1, IL33, IRF7, SERPINE1, SPP1, STAT1, STAT3, UCP2 and VCAM1 (Fig. 5).

Molecular network analysis. The interaction network analysis shows the interactions between molecules in the dataset. All networks were then sorted using the score values. The highest ranked network (score 52) was found to mainly affect ‘neurological disease, hereditary disorder, organismal injury and abnormalities’, involving 35 molecules (ACTR3, DNPPEP, DPYSL3, DUS2, DYSF, ECH1, ENY2, EPRD1, EPRS, ERF, FAM136A, Flt1, HNRNPU1, HPRT1, KARS, LEPRTL1, MAG, MAPK3, MARS, MRPS16, MYRF, NF2, PRPF40A, RBFOX2, RERE, Rho gdi, SCAND1, SDCBP, SERTAD2, TCEAL9, TNKS2, TPR, TSN, XPNPEP3, and ZBTB14; Table SVIII). The associated interaction network map in terms of these aforementioned molecules is shown in Fig. 6. The network ranked at no. 19 affects ‘cardiovascular disease’ and ‘cardiovascular system development and function’, which involves 35 molecules (Arf, ARF3, Calcineurin A, CMIP, elastase, ELF2, Ephb, EPHB4, ETS, FKBP2, FKBP1A, HMMR, NCK, NR2F2, OAF, PABPN1, PAI2, PAPOLA, Pde4, peptidylprolyl isomerase, Pnmc, PMEPAI, PPIC, PURB, RNF111, Smad, SMAD6, Smad1/5/8, Smad2/3-Smad4, Smad2/3, TCF23, TGFB1, TM2D3, Vegf, and Wasp; Table SVIII).

Validation of target genes. In total, 20 of the genes of interest were selected for qPCR validation and 10 protein products were verified by western blotting. These genes were chosen from the list of enriched canonical pathways, a proportion of which were found to be the DEGs. Since H9C2 cells with RhoE-knockout proliferate slowly (data not shown), genes associated with cell cycle were also subjected to validation. The expression levels of the genes that were found to be upregulated after RhoE-knockout (ELK1, FTH1, ADAM10, and TNFRSF11b) and the genes that were downregulated (TIMP3, IL6st, SMC1a, CCNB1, CCND2, CCNA1, SHC1, CCXL12, JAK2, CDK6, CBL, and DUSP1; Table SVIII).

Discussion

To investigate the global gene profile affected by alterations in RhoE expression, RhoE-knockout and wild-type H9C2 cardiomyocytes were used as models to detect DEGs and
identify the potential associated underlying signaling pathways. Achieving RhoE-knockout in H9C2 cells was a critical step, since obtaining RhoE-knockout cardiomyocytes in vivo is difficult because of the low efficiency (<0.9%) in establishing adult RhoE−/- mice due to the high embryonic lethality after RhoE-knockout (10). Therefore, the powerful genome editing system CRISPR/Cas9 was applied to establish RhoE-knockout in H9C2 cells (28,29), which was ultimately successful. However, establishing a monoclonal H9C2 RhoE-knockout cell line was not possible due to the slow proliferative characteristics of the cells. Therefore, puromycin-stressed H9C2 cells of mixed clones was used to test the genome editing efficiency. Cells from the puromycin-stressed cell pools display heterogeneous genotypes in the mixed cell clones, thus, the mixed cell clones were not available for DNA sequencing. However, western blotting results confirmed that expression of the RhoE protein was significantly reduced after RhoE gene editing. Therefore, lentiviruses encoding the sgRNA-1 sequence and Cas9 were used for subsequent experiments.

After CRISPR/Cas9-mediated RhoE-knockout, potential DEGs were detected in the RhoE-knockout and wild-type H9C2 cells. The cells were first infected with the lentiviruses containing sgRNA-1 and Cas9 at a high multiplicity of infection of 100. Total RNA was then extracted and subjected to chip analysis. A total of 829 differential expressing genes were found, where 417 were upregulated and 412 were downregulated. The top five upregulated genes were LOC100912383, TNN, CLU, IL33 and ATP7A, whilst the top five downregulated genes were LOC100912383, TNN, CLU, IL33 and ATP7A.
Table I. A list of 30 representative upstream regulators of RhoE.

<table>
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<tr>
<th>Upstream regulator</th>
<th>Molecule type</th>
<th>Predicted activation state</th>
<th>z-score</th>
<th>P-value of overlap</th>
<th>Target molecules in dataset</th>
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</thead>
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<td>-3.002</td>
<td>2.17x10^{-08}</td>
<td>ACSS2, CYP51A1, DHC7, ELOVL6, FASN, FDFT1, FDPS, HMGCR, IDI1, INSIG1, LDLR, LSS, MSMO1, MVD, MVK, NSDHL, OGDH, PD4, PD4, PDK4, SC5D, SERPINE1, SOCS3, SQLE, UCP2, CAM1-</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>Chemical drug</td>
<td>Inhibited</td>
<td>-2.942</td>
<td>3.84x10^{-07}</td>
<td>ACSS2, CYP51A1, DHC7, ELOVL6, FASN, FDFT1, FDPS, HMGCR, IDI1, INSIG1, LDLR, LSS, MSMO1, MVD, MVK, NSDHL, OGDH, PD4, PD4, PDK4, SC5D, SERPINE1, SOCS3, SQLE, UCP2, CAM1-</td>
</tr>
<tr>
<td>ATP7B</td>
<td>Transporter</td>
<td>Inhibited</td>
<td>-2.887</td>
<td>7.03x10^{-09}</td>
<td>ACSS2, CYP51A1, DHC7, ELOVL6, FASN, FDFT1, FDPS, HMGCR, IDI1, INSIG1, LDLR, LSS, MSMO1, MVD, MVK, NSDHL, OGDH, PD4, PD4, PDK4, SC5D, SERPINE1, SOCS3, SQLE, UCP2, CAM1-</td>
</tr>
<tr>
<td>Itavastatin</td>
<td>Chemical drug</td>
<td>Inhibited</td>
<td>-2.800</td>
<td>5.59x10^{-07}</td>
<td>ACSS2, CYP51A1, DHC7, ELOVL6, FASN, FDFT1, FDPS, HMGCR, IDI1, INSIG1, LDLR, LSS, MSMO1, MVD, MVK, NSDHL, OGDH, PD4, PD4, PDK4, SC5D, SERPINE1, SOCS3, SQLE, UCP2, CAM1-</td>
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Table I. Continued.

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<tr>
<th>Upstream regulator</th>
<th>Molecule type</th>
<th>Predicted activation state</th>
<th>Activation z-score</th>
<th>P-value of overlap</th>
<th>Target molecules in dataset</th>
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<tr>
<td>Pirinixic acid</td>
<td>Chemical toxicant</td>
<td>Inhibited</td>
<td>-2.661</td>
<td>$3.37 \times 10^{-11}$</td>
<td>ABCC1, ACSS2, AK1C3, ASNS, BSG, CCNB1, CCND1, CCT6A, CDK1A, CBF1, CLU, CXCL2, CYP7A1, DUSP1, ELOVL6, FADS1, FASN, FKB1A, Gk, GSTM3, HMGR, HMgn2 (includes others), ID2, IFTM3, INSIG1, LDLR, LGALS3, MARK2, MLYCD, NBN, PDCD4, PDK4, PIK3R3, PLA2G2A, PPIC, PSMB4, RINGX2A, RORA, RTN4, SC5D, SCD, SERPINE1, UQBLN2, UCP2, VCAM1, Yap1</td>
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<tr>
<td>PPARGC1B</td>
<td>Transcription regulator</td>
<td>Inhibited</td>
<td>-2.614</td>
<td>$5.72 \times 10^{-97}$</td>
<td>CCND1, COX41, FASN, FDF1T1, FDP1, HMGR, LSS, MVD, MVK, PDK4, SCD, SCDQ, SLE</td>
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<tr>
<td>Decitabine</td>
<td>Chemical drug</td>
<td>Activated</td>
<td>2.724</td>
<td>$1.57 \times 10^{-06}$</td>
<td>ABCC1, AGRN, AKAP12, BCL2L11, BTG1, CCND1, CCND2, CD44, CDKN1A, CLU, COL6A3, CXCL12, CYP1B1, DUSP1, EPHB4, GSN, HMGR, HPRT1, HSPA1A, HSPA1B, ID1, IFT7, ISG15, JAG1, KLF6, KRT7, LDLR, LGALS3, MMP14, NDK, SGK1, SHC1, SMARCA2, SOCS3, SPP1, STAT1, TFAP2A, TGFβ1, TIMP3, Tpm3, UBA1</td>
</tr>
<tr>
<td>IFNAR1</td>
<td>Transmembrane receptor</td>
<td>Activated</td>
<td>2.760</td>
<td>$2.02 \times 10^{-02}$</td>
<td>ELOVL6, FASN, FDFT1, FDP1, HMGR, INSIG1, LDLR, SCD</td>
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<tr>
<td>INSIG2</td>
<td>Other</td>
<td>Activated</td>
<td>2.764</td>
<td>$1.41 \times 10^{-06}$</td>
<td>CXCL2, CYP7A1, FASN, HMGR, J133, LDLR, SCD, VCAM1</td>
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<tr>
<td>RIPK2</td>
<td>Kinase</td>
<td>Activated</td>
<td>2.764</td>
<td>$3.60 \times 10^{-03}$</td>
<td>CDKN1A, COL6A1, CXCL12, DIXDC1, MOY, SERPINE1, SLC44A1, Tpm3, ZEB1</td>
</tr>
<tr>
<td>SKIL</td>
<td>Transcription regulator</td>
<td>Activated</td>
<td>2.779</td>
<td>$2.23 \times 10^{-05}$</td>
<td>CXCL12, CYP5A1, DHCR7, FASN, FSTL1, HMGR, LDLR, MMR4, PRRX2, SCD, SLE, TGFβ1, WISP2</td>
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<tr>
<td>LMNB1</td>
<td>Other</td>
<td>Activated</td>
<td>2.804</td>
<td>$2.07 \times 10^{-09}$</td>
<td>CYP7A1, DHCR7, FDF1T1, FDP1, HMGR, J131, LSS, MVK, PMVK</td>
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<tr>
<td>PEX5L</td>
<td>Ion channel</td>
<td>Activated</td>
<td>2.970</td>
<td>$6.14 \times 10^{-11}$</td>
<td>ADAM10, ADAR, AGRN, AXL, CD14, CD44, CD47, CXCL2, DUSP1, ETNK1, IFIT5, IRF7, ISG15, JAK2, KLF6, PRKAR 1B, RC3H2, STAT1, TIPARP, TNFRSF11B, Traf6, TRIM25, VCAM1</td>
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<tr>
<td>Poly rI: rC - RNA</td>
<td>Biologic drug</td>
<td>Activated</td>
<td>2.994</td>
<td>$4.49 \times 10^{-02}$</td>
<td>AGRN, ARNT2, ASNS, ATG3, ATP1B1, ATP2A2, BCL2L11, BTG1, C1R, CCND1, CCND2, CD44, CDKN1A, CRBP1, CXADR, CXCL12, CXCL2, DDR2, DRG1, DUSP1, ECE1, ELK1, ETSA, FASN, FHL2, FTH1, GAS6, GIA5, HMGR, HSPA1A, HSPA1B, ID1, IFT7, ISG15, JAGA6, ITGB1, JAK1, JAK2, KARS, KLF6, LAMC2, LGALS3, MMP11, MTSS1, MOY, NDRG4, PAM, PHGDH, PLA2G2A, PRAKCA, RAB12, SBN2O2, SDC1, SERPINE1, SLC12A2, SLC1A3, SLC4A2, SLC7A5, SMTN, SOCS3, SOD3, SPP1, SQU, ATP3, STAT1, STAT3, STAT6, TBC1D10A, TGFβ2, TIMP3, TNFRSF11B, Traf6, UNC5B, VCAM1, ZYX</td>
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<tr>
<td>IFNG</td>
<td>Cytokine</td>
<td>Activated</td>
<td>3.098</td>
<td>$1.58 \times 10^{-05}$</td>
<td>AGRN, ARNT2, ASNS, ATG3, ATP1B1, ATP2A2, BCL2L11, BTG1, C1R, CCND1, CCND2, CD44, CDKN1A, CRBP1, CXADR, CXCL12, CXCL2, DDR2, DRG1, DUSP1, ECE1, ELK1, ETSA, FASN, FHL2, FTH1, GAS6, GIA5, HMGR, HSPA1A, HSPA1B, ID1, IFT7, ISG15, JAGA6, ITGB1, JAK1, JAK2, KARS, KLF6, LAMC2, LGALS3, MMP11, MTSS1, MOY, NDRG4, PAM, PHGDH, PLA2G2A, PRAKCA, RAB12, SBN2O2, SDC1, SERPINE1, SLC12A2, SLC1A3, SLC4A2, SLC7A5, SMTN, SOCS3, SOD3, SPP1, SQU, ATP3, STAT1, STAT3, STAT6, TBC1D10A, TGFβ2, TIMP3, TNFRSF11B, Traf6, UNC5B, VCAM1, ZYX</td>
</tr>
<tr>
<td>Upstream regulator</td>
<td>Molecule type</td>
<td>Activation state</td>
<td>z-score</td>
<td>P-value of overlap</td>
<td>Target molecules in dataset</td>
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<tr>
<td>IKBKG</td>
<td>Kinase</td>
<td>Activated</td>
<td>3.240</td>
<td>5.90x10^{-03}</td>
<td>ATP2A2,CLU,CXCL12,CXCL2,IRF7,ISG15,NID1,SERPINE2,SGK1,SOCS3,SOD3</td>
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<tr>
<td>CD28</td>
<td>Transmembrane receptor</td>
<td>Activated</td>
<td>3.320</td>
<td>1.40x10^{-02}</td>
<td>ACTR3,AP1B1,B'TG1,CBL,CBLB,CCND1,CCND2,CXCL2,FDPS,FKB2,HAT1,HSPA1A/HSPA1B,IGFBP5,PSMF1,RAD23B,RORA,SLC7A5,SOCS3,STAT3,STEAP2,STRN3</td>
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<tr>
<td>MAPK14</td>
<td>Kinase</td>
<td>Activated</td>
<td>3.376</td>
<td>7.84x10^{-04}</td>
<td>CCNB1,CCND1,CYP51A1,CYP7A1,DUSP1,FDPS,HMGCR,LDLR,MYOG,SGK1,SHC1,SLC18A3,SOCS3,TNN,Tpm3</td>
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<tr>
<td>INSIG1</td>
<td>Other</td>
<td>Activated</td>
<td>3.627</td>
<td>1.04x10^{-11}</td>
<td>ACSS2,CXCL2,CYP51A1,DHCR7,ELOV1F,FDAS1,FDPS,HMGCR,INSIG1,LDLR,PSMF1,RAD23B,RORA,SLC7A5,SOCS3,STAT3,STEAP2,STRN3</td>
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<tr>
<td>POR</td>
<td>Enzyme</td>
<td>Activated</td>
<td>3.754</td>
<td>3.97x10^{-13}</td>
<td>ABCA7,ACSS2,BSG,CD14,CXCL2,CYP7A1,DHCR7,FDAS1,FDPS,HMGCR,INSIG1,LDLR,PMVK,SCD,Scd2,SQLE,STAT1,TM7SF2,TMEM97</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Chemical- endogenous</td>
<td>Activated</td>
<td>4.423</td>
<td>5.86x10^{-12}</td>
<td>ABCA7,ACSS2,BSG,CD14,CXCL2,CYP7A1,DHCR7,FDAS1,FDPS,HMGCR,INSIG1,LDLR,PMVK,PSAT1,PLP36AL,SCD,STAT1,TM7SF2,TMEM97,VCAM1</td>
</tr>
</tbody>
</table>
five downregulated genes were WAS1, HIST1H1D, IDI1, SCD1 and TBL1X. LOC100912383 is an unannotated rat gene that encodes a protein with unknown function. The other nine genes are known protein-encoding genes with Gene ontology (http://geneontology.org/) and GeneCards (https://www.genecards.org/) annotations revealing associated functions, related pathways and associated diseases. Interestingly, a PubMed search indicated that the majority of the top five up- and downregulated genes were associated with cardiovascular system diseases (30-34). The DEGs found in the present study will facilitate the discovery of the global properties of RhoE further.

The IPA system can be applied to integrate gene expression, microRNA and small-scale experimental data (20,21). It establishes a visualized experimental system that reveals the properties of molecules, including genes, proteins, chemicals and drugs, along with the network of interactions between these molecules. The present study applied the IPA system to uncover the signaling pathways, interactions and functional roles of DEGs associated with RhoE-knockout. The module of the canonical pathway analysis related to disease and function, regulator effects, upstream regulators and molecular networks was focused upon.

The results of canonical pathway analysis indicated that RhoE may be involved in 67 canonical pathways with ‘cholesterol biosynthesis’ superpathway found to be the highest ranking signaling pathway, suggesting the potential role of RhoE in cholesterol biosynthesis. In addition, ‘androgen signaling’, ‘interferon signaling’, ‘Nrf2-mediated oxidative stress response’, ‘Gβγ signaling’, ‘oncostatin M signaling’ and ‘thrombopoietin signaling’ were also revealed to be significantly activated. In total, 40 signaling pathways yielded Z-scores ≥1 but none >2. The ‘osteoarthritis pathway’, ‘Wnt/β-catenin signaling’, ‘TGF-β signaling’, ‘regulation of actin-based motility by Rho’, ‘NF-κB signaling’, ‘Cdc42 signaling’ and ‘glioma invasiveness signaling’ and ‘signaling
by Rho family GTPases’ were found to be inhibited. Among the 67 enriched canonical pathways, ‘NF-κB signaling’, ‘oncostatin M signaling’, ‘ERK/MAPK signaling’, ‘leukocyte extravasation signaling’, ‘IL-3 signaling’, ‘GM-CSF signaling’, ‘TGF-β signaling’, ‘Interferon signaling’ and ‘IL-22 signaling’ were associated with the inflammatory response. Subsequent PubMed search found three articles that reported a relationship between RhoE and NF-κB signaling (12,35,36), especially one recently published by Dai et al (12), which elegantly proved that RhoE deficiency led to the hyperactivation of NF-κB, resulting in the recruitment of inflammatory cells post myocardial infarction. Activation of NF-κB signaling serves a critical role in a number of human diseases, including that in the cardiovascular system (37,38), the RhoE/NF-κB signaling

Figure 5. Network diagram representing the regulatory effects with top consistency scores. After RhoE-knockout, the top network, which yielded a consistency score=8.043, was ‘adhesion of endothelial cells, recruitment of myeloid cells’. Possible regulatory pathways regulating the recruitment of myeloid cells and adhesion of endothelial cells are displayed.

Figure 6. Gene interaction network map. This network consists of the top ranked network found associated with the role of RhoE in neurological disease, hereditary disorder and organismal injury and abnormalities.
The large number of signaling pathways that were found to be enriched in the present study, which can potentially provide a resource for exploring human disease intervention strategies using RhoE as a molecular target.

Upstream regulator analysis detected 885 upstream regulators that were enriched. Cholesterol was identified as the most powerful activator whereas SCAP was the most powerful inhibitor. Rosuvastatin, atorvastatin, lovastatin and pitavastatin are drugs that have been widely used for the treatment of cardiovascular diseases (39). Interestingly, these four statins were found to be enriched and were potential inhibitors, all with Z-scores < -2. The upstream regulators detected in the present study provide a reference for elucidating the mechanism of molecular disorders in specific diseases that are associated with aberrant RhoE expression.

The disease and functional analysis indicated further that RhoE has multifaceted functionality. Organismal survival was demonstrated to be in the top ranking category, which had annotations related to morbidity or mortality, with 241 associated molecules that were enriched. The absence of RhoE strongly predicted an impairment in this function, with a Z-score of -2.362. A previous report indicated that the long noncoding RNA TUG1 modulated the proliferation of trophoblast cells via the epigenetic suppression of RhoE, leading to the onset of pre-eclampsia (40), thereby highlighting the function of RhoE in organismal survival. There were numerous reports on the function of RhoE in cancer, organismal injury and abnormalities. For cancers of the digestive system (41-43), breast (44), prostate (45) and brain (46), RhoE has been previously identified as a tumor suppressor. However, RhoE has also been documented to serve as a tumor promoter in gastric and lung cancers (47,48). The role of RhoE in cancer seems to be dependent on the cell type (5). In the present study, RhoE was also revealed to serves roles in a total of 36 aspects of cardiovascular disease and development function. Binding of endothelial cells and adhesion of endothelial cells are the two processes that were predicted to be strongly increased, whilst angiogenesis and vasculogenesis are predicted to be reduced even though the Z-scores were lower. Breslin et al (13) provided evidence that supports RhoE function in vascular endothelial barrier recovery and Yue et al (8) proposed that RhoE deficiency suppressed angiogenesis in heart failure. These aforementioned previous reports suggest that RhoE serves a role in cardiovascular diseases and has potential value for targeted interventions. Apart from cardiovascular diseases and function, RhoE may also regulate ‘cell cycle progression’, ‘cell-to-cell signaling and interaction’, ‘cellular function and maintenance’, and ‘molecular transport’, which further demonstrate the versatility of RhoE.

A total of 33 kinds of regulatory effects were predicted using the IPA system. Adhesion of endothelial cells and recruitment of myeloid cells were thought to be the top effects after RhoE-knockout. Additionally, ~33% of the total regulatory effects were associated with cancer, followed by inflammation. Taken together, the DEGs established a total of 25 regulatory networks. The top network, ranked based on scores, mainly affects ‘neurological disease, hereditary disorder, organismal...
involving the differential molecules after RhoE- 
latory effects and networks revealed the major interactions 
involving 34 molecules. The regu 
oxidative response, Cholesterol signaling, Eph-Ephrin 
cluding those in oncostatin M signaling, Nrf2-mediated 
these genes belong to a number different signaling pathways, 
cluding 34 molecules. The regu 
10 proteins for western blotting validation, which found that 
slowly after 
help explain the phenomenon that the H9C2 cells proliferated 
CDK6 
ative models should be established to verify these 
 limitations associated with the present study: (i) Specific 
according to the IPA analysis, further research is 
related to the mechanisms involving the differential 
 signalling pathways and molecular networks that are specific 
to cardiovascular diseases; (iii) Due to the inherent defects of 
CRISPR/Cas9 technology, the potential off-targets effects of 
gene in H9c2 cells using cRISPR/cas9 technology, which 
other genome editing methods can be used to verify the 
results of this study.

In conclusion, the present study knocked out the RhoE 
gene in H9C2 cells using CRISPR/Cas9 technology, which 
obtained the RhoE-knockout cells. Subsequently, a set of 
DEGs between RhoE-knockout and wild-type H9C2 cells 
was obtained using genome-wide expression microarrays. 
Using the microarray data, the potential canonical signaling 
pathways and the associated diseases and function, upstream 
regulatory molecules, regulatory effects and interaction 
networks involving the DEGs as a result of RhoE-knockout 
were enriched and analyzed using the IPA system. RhoE was 
speculated to regulate a multitude of pathways, including 
the superpathway of cholesterol biosynthesis, oncostatin M 
signaling, interferon signaling and regulation of actin-based 
motility by Rho. In addition, data from the present study also 
suggest that RhoE serves a role in diseases and functions, 
including organismal survival, cancer, cardiovascular disease 
and development function. The present study uncovered some 
of the previously unknown global biological and functional 
properties of RhoE.

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Availability of data and materials

The datasets used and/or analyzed during the current study are 
available from the corresponding author on reasonable request.

Authors' contributions

ZShao, KW, SZ, JY, XL, CW, YZ and YH performed the experiments and collected data. ZShen searched literature and performed statistical analyses. WJ and JG conceived and coordinated the study and drafted the manuscript. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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