

**NR4A2 inhibits activation of ERK signaling and cell growth  
in response to beta-adrenergic stimulation in adult rat cardiomyocytes**

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Running Head: Adult cardiomyocytes growth inhibition by NR4A2

**SUPPLEMENTAL FIGURE CAPTIONS**

Supplemental Figure S1. Time course depicting cell manipulations (Transduction and pharmacological treatment) with subsequent analyses for the assessment of gene expression levels, signal transduction activity, and cell physiological parameters in response to those manipulations. ISO, isoproterenol treatment (10  $\mu\text{mol/L}$ )

Supplemental Figure S2. Generation of highly pure cultures of adult cardiomyocytes. *A*: Representative visualization by immunofluorescence of ARVMs after 96 hrs in culture. Most cardiomyocytes maintained their rod-shaped structure at this time. *B*: Real-time PCR quantification of mRNAs encoding cardiac myocyte markers (Myh6, Myh7, Tnnt2) and fibroblast markers (Vim, Col1a1, Col1a2) in the ARVMs and non-myocyte control cell fraction after 96 hrs in culture. Data are means  $\pm$  SE of three independent experiments. Pairwise comparison was performed with unpaired Student's *t*-test.  $*P < 0.05$  vs. non-myocyte fraction. *C*: Immunodetection in the ARVMs and non-myocyte control cell fraction of markers specific to endothelial cells (RECA-1), fibroblasts (Vimentin), smooth muscle cells (Sm22- $\alpha$ ) and macrophages (CD68). Representative data from three independent experiments. *D*: RNA-seq analysis-FPKM values for cardiac cell-specific markers (myocyte, endothelial, fibroblast, neutrophil, macrophage, and lymphocyte). The markers and their respective FPKM values were mined from the RNA sequencing data obtained for GFP expressing control ARVMs presented in Fig. 3. Data are means  $\pm$  SE of six independent experiments.

Supplemental Figure S3. NR4A2 is induced by isoproterenol in H9c2 cardiomyoblasts and inhibits cell growth response to the beta-adrenergic agonist. *A*: Time course of NR4A2 mRNA expression level in response to 10  $\mu\text{mol/L}$  isoproterenol. Data are means  $\pm$  SE of three independent experiments. Interaction between multiple groups was determined by one-way ANOVA, including a Tukey's post hoc analysis when significant interaction occurred.  $*P < 0.05$  vs. baseline.  $^{\dagger}P < 0.05$  vs. time 1hr. *B*: Quantification of cell surface area in response to a 48-hour treatment with 10  $\mu\text{mol/L}$  isoproterenol in non-transduced (NTD), GFP expressing, and NR4A2 overexpressing H9c2 cells visualized by phalloidin conjugate staining of actin filaments. Data are means  $\pm$  SE of three independent experiments. Interaction between multiple groups was determined by one-way ANOVA, including a Newman-Keuls' post hoc analysis when significant interaction occurred.  $*P < 0.05$  vs. vehicle.  $^{\dagger}P < 0.05$ .

**Supplemental Table S1.** Primers and probes used for real-time PCR assays. All assays designed to match rat gene sequences.

Gene Name	Gene Symbol	Primer	Sequence
Nuclear Receptor Subfamily 4 Group A Member 2 <sup>1</sup>	Nr4a2	Forward Reverse Probe	5'-ACATTTCTGCCTTCTCCTG-3' 5'-TTGTAGCTCTTCCACTCTC-3' 5'-FAM-AGCCCGTGTCTCTCTGTGACCAT-TAMRA-3'
Natriuretic Peptide B	Nppb	Forward Reverse Probe	5'-CTGCTTTTCCTTAATCTGTGCG-3' 5'-GCCATTTCTCTGACTTTTC-3' 5'-FAM-TGCATCGTGGATTGTTCTGGAGACTG-TAMRA-3'
Troponin T2, Cardiac Type	Tnnt2	Forward Reverse Probe	5'-AGACAGGATCGAAAAGCG-3' 5'-GCCTCATCTTCAGCCTTC-3' 5'-FAM-TGCCTTTCTCTCTCGTTTATTGCG-TAMRA-3'
Myosin Heavy Chain 6	Myh6	Forward Reverse Probe	5'-TTCTCCACCTATGCTTCTG-3' 5'-GCTTGTTTCAAGTTTCCCG-3' 5'-FAM-TCCTTTGCCTTTCCCACTGTCACC-TAMRA-3'
Myosin Heavy Chain 7	Myh7	Forward Reverse Probe	5'-GCCTCAATAGACTCTGAAG-3' 5'-TCCCTCTGCTTCTGTTG-3' 5'-FAM-CCTTTGATGTGCTGGGCTTCACTCC-TAMRA-3'
Collagen Type I Alpha 1 Chain	Col1a1	Forward Reverse Probe	5'-CCCCAAAGACACAGGAAATA-3' 5'-CCTTCAATAGTCCGAGAACC-3' 5'-FAM-TCCCTACCCAGCACCTTCAAATCCT-TAMRA-3'
Collagen Type I Alpha 2 Chain	Col1a2	Forward Reverse Probe	5'-GGTCCTCATGGTTCTGTG-3' 5'-CCCTTGTCACCTCGAATAC-3' 5'-FAM-TGCTGGTTCTGTTGGTCCCGTTG-TAMRA-3'
Vimentin	Vim	Forward Reverse Probe	5'-AGAACACTCCTGATTAAGACG-3' 5'-TTTTATTCAAGGTCATCGTGG-3' 5'-FAM-CAGAGACGGACAGGTGATCAATGAGACT-TAMRA-3'
Glyceraldehyde-3-Phosphate Dehydrogenase	Gapdh	Forward Reverse Probe	5'-GCTGCCTTCTCTTGTGAC-3' 5'-CCAGCTTCCCATTCTCAG-3' 5'-FAM-ACGACCCCTTCATTGACCTCAACTACA-TAMRA-3'
Dual Specificity Phosphatase 2	Dusp2	Forward Reverse Probe	5'-GAAAATAACAGCTCTGACCCG-3' 5'-GAGACATTGAGAACCGCTG-3' 5'-FAM-AGGGCAAGATTTCCACAGGACCAC-TAMRA-3'
Dual Specificity Phosphatase 14	Dusp14	Forward Reverse Probe	5'-ACTGTTCTTGCCTTCACTCTG-3' 5'-TCTTCCCTAAACGTCTGTCC-3' 5'-FAM-TGGCCCTGATTAACCCTCTGGAGAC-TAMRA-3'
Cyclophilin A	Ppia	Forward Reverse Probe	5'-CTGATGGCGAGCCCTTG-3' 5'-TCTGCTGTCTTTGGAACCTTGTC-3' 5'-FAM-CGCGTCTGCTTCGAGCTGTTTGCA-TAMRA-3'

18S ribosomal RNA	RNA18S	Forward	5'-GGCTTAATTTGACTCAACACG-3'
		Reverse	5'-CGGAATCGAGAAAGAGCTATC-3'
		Probe	5'-FAM-CGGACACGGACAGGATTGACAGATTG-TAMRA-3'

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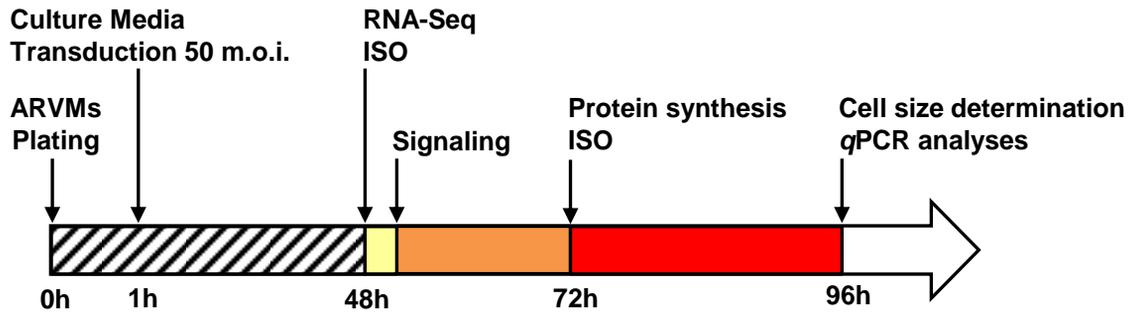
<sup>¶</sup> The primers and probe for the rat cDNA sequence of NR4A2 have >96% homology with the human NR4A2 cDNA sequence and were used to simultaneously detect mRNA levels from both species in Ad-h-NR4A2 transduced cells.

**Supplemental Table S2.** Primary antibodies used in immunofluorescence and Western blotting analyses.

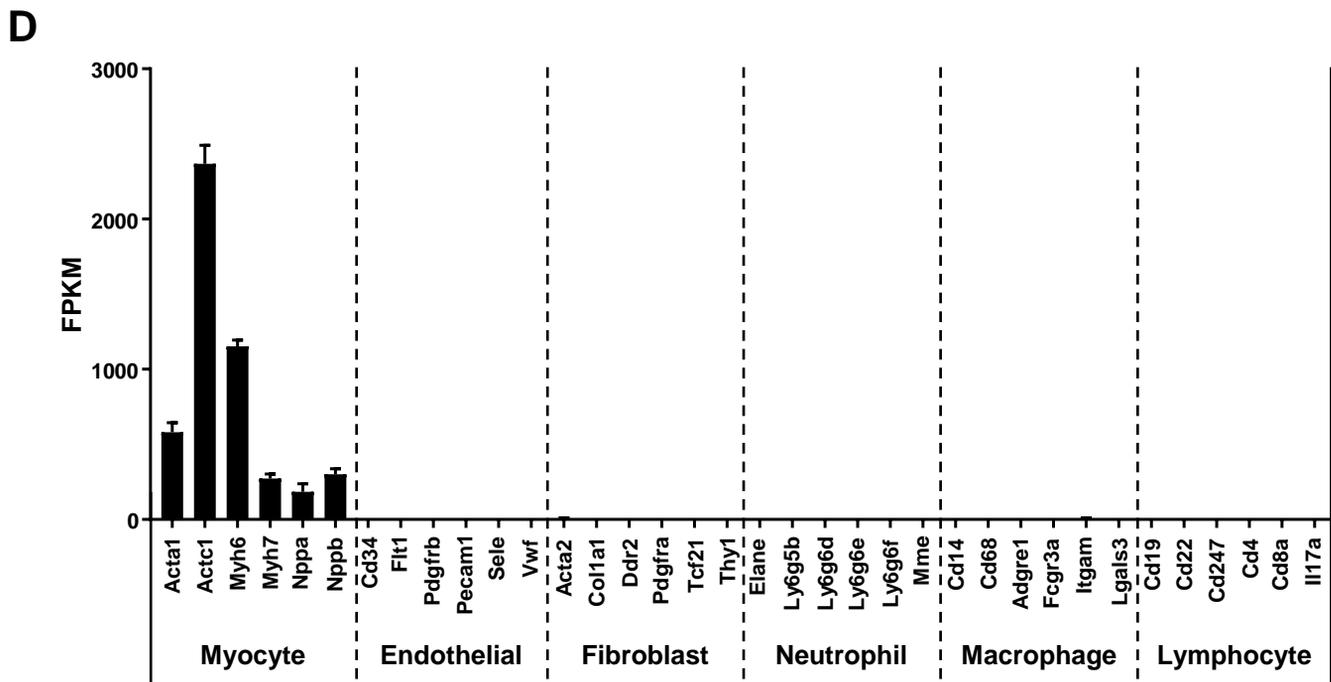
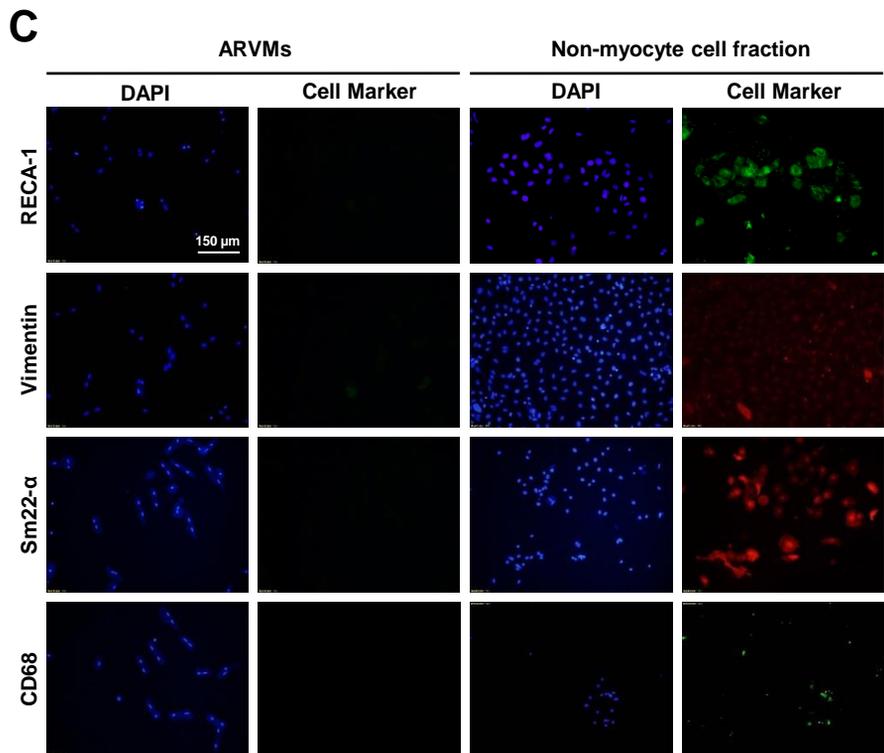
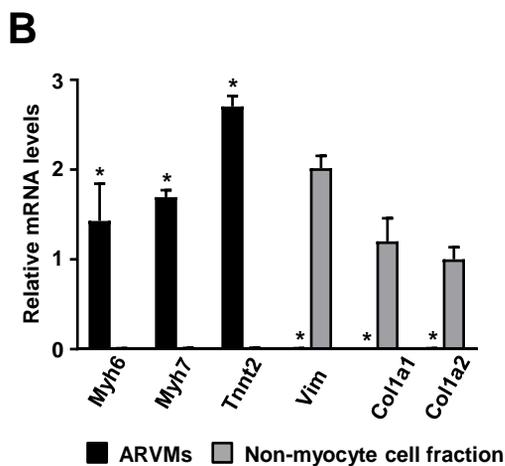
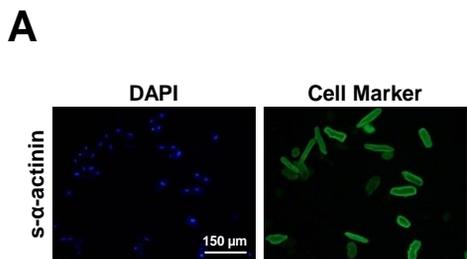
Target Protein	Antibody Full Commercial Name	Vendor	Catalog No.	RRID	Host Species	Application	Concentration Used
Akt	Akt1/2/3 (H-136)	SC	Sc-8312	AB_671714	Rabbit	WB	1:1000
Phospho-Akt (S473)	Phospho-Akt (Ser473) (193H12)	CST	#4058	AB_331168	Rabbit	WB	1:500
Alpha-Actinin	Anti-Sarcomeric Alpha Actinin antibody [EA-53]	Ab	ab9465	AB_307264	Mouse	IF	1:100
CD68	Mouse anti Rat CD68	BR	MCA341GA	AB_566872	Mouse	IF	1:100
CREB	Anti-CREB antibody [LB9]	Ab	Ab178322	N/A	Mouse	WB	1:1000
Phospho-CREB (S133)	Anti-CREB (phospho S133) antibody [E113]	Ab	Ab32096	AB_731734	Rabbit	WB	1:1000
Erk1/2	P44/42 MAPK (Erk1/2) (137F5)	CST	#4695	AB_390779	Rabbit	WB	1:1000
Phospho-Erk1/2 (T202/Y204)	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2)	CST	#4377	AB_331775	Rabbit	WB	1:500
GSK-3 $\beta$	GSK-3 $\beta$ (27C10)	CST	#9315	AB_490890	Rabbit	WB	1:1000
Phospho-GSK-3 $\beta$ (S9)	Phospho-GSK-3 $\beta$ (Ser9) (5B3)	CST	#9323	AB_2115201	Rabbit	WB	1:1000
HSP60	HSP60 (D6F1) XP®	CST	#12165	AB_2636980	Rabbit	WB	1:1000
NR4A2	Anti-Nurr1 antibody [N1404] – ChIP Grade	Ab	Ab41917	AB_776887	Mouse	IF WB	1:100 1:500
p70 S6 Kinase	p70 S6 Kinase (49D7)	CST	#2708	AB_390722	Rabbit	WB	1:1000
Phospho-p70 S6 Kinase (T389)	Phospho-p70 S6 Kinase (Thr389) (108D2)	CST	#9234	AB_2269803	Rabbit	WB	1:500
Rat Endothelial Cell Antigen	Anti-Endothelial Cell antibody [RECA-1]	Ab	Ab9774	AB_296613	Mouse	IF	1:100
SM22 alpha	Anti-SM22 alpha antibody	Ab	ab14106	AB_443021	Rabbit	IF	3 $\mu$ g/ml
Troponin T	Anti-Cardiac Troponin T antibody	Ab	Ab45932	AB_956386	Rabbit	IF	1:400
Vimentin	Anti-Vimentin antibody [RV202]	Ab	Ab8978	AB_306907	Mouse	IF	1:100

Ab, Abcam; BR, Bio-Rad; CST, Cell Signaling Technology; IF, immunofluorescence; SC, Santa Cruz Biotechnology; WB, Western blotting.

# Supplemental Figure S1

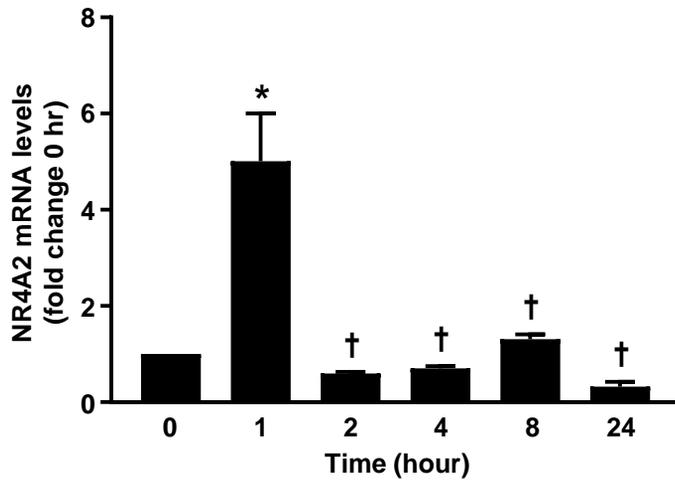


# Supplemental Figure S2



# Supplemental Figure S3

A



B

