Long Non-coding RNA ECRAR Triggers Post-natal Myocardial Regeneration by Activating ERK1/2 Signaling

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Reactivating post-natal myocardial regeneration potential may be a feasible strategy to regenerate the injured adult heart. Long non-coding RNAs (lncRNAs) have been implicated in regulating cellular differentiation, but whether they can elicit a regenerative response in the post-natal heart remains unknown. In this study, by characterizing the lncRNA transcriptome in human hearts during the fetal-to-adult transition, we found that 3,092 lncRNAs were differentially expressed, and we further identified a novel upregulated fetal lncRNA that we called endogenous cardiac regeneration-associated regulator (ECRAR), which promoted DNA synthesis, mitosis, and cytokinesis in post-natal day 7 and adult rat cardiomyocytes (CMs). Overexpression of ECRAR markedly stimulated myocardial regeneration and induced recovery of cardiac function after myocardial infarction (MI). Knockdown of ECRAR inhibited post-natal day 1 CM proliferation and prevented post-MI recovery. ECRAR was transcriptionally upregulated by E2F transcription factor 1 (E2F1). In addition, ECRAR directly bound to and promoted the phosphorylation of extra-cellular signal-regulated kinases 1 and 2 (ERK1/2), resulting in downstream targets of cyclin D1 and cyclin E1 activation, which, in turn, activated E2F1. The E2F1-ECRAR-ERK1/2 signaling formed a positive feedback loop to drive cell cycle progression, and, therefore, it promoted CM proliferation. These findings indicated that our newly discovered ECRAR may be a valuable therapeutic target for heart failure.

INTRODUCTION

In response to ischemic injury, the adult human heart has a limited capacity for regeneration to compensate for lost myocardial tissue. The human heart typically responds to injury through a scarring mechanism rather than through myocardial regeneration, which results in adverse remodeling and subsequent progression to heart failure. Therefore, an understanding of the underlying mechanisms and ability to stimulate the regenerative capacity of the human heart is of the utmost scientific and clinical importance. The adult hearts of lower vertebrates, such as zebrafish and amphibians, have shown robust regenerative capacity. In mammals, fetal cardiomyocytes (CMs) can proliferate robustly with heart growth primarily occurring by hyperplasia. Until 7 days after birth, the neonatal rodent heart maintains a significant capacity for cardiac repair and regeneration after injury. It was recently reported that human newborns also show an intrinsic capacity to repair myocardial damage and recover cardiac function completely. However, the endogenous regeneration capacity of the mammalian heart is largely lost by 7 days after birth, and it remains absent throughout the lifespan. It is unclear why this robust regenerative response at birth is lost with age. Exploring the mechanisms that underlie this fetal-to-adult heart transition may reveal new therapeutic strategies to reactivate post-natal myocardial regeneration in the adult heart after injury.

Long non-coding RNAs (lncRNAs) are a novel class of transcripts, larger than 200 nt and with poor protein-coding potential, that can regulate gene expression at the epigenetic, transcriptional, and post-transcriptional levels. Increasing evidence has indicated that lncRNAs are differentially expressed across various tissues, diseases, and developmental stages and are key regulators in a wide range of biological processes, including cell proliferation, apoptosis, cell cycle control, and cell differentiation. lncRNAs are emerging as important players in heart development, heart failure, CM hypertrophy, and atherosclerosis. A recent study revealed that lncRNAs have tissue-specific expression, and the authors identified 321 cardiac-expressed lncRNAs and 52 cardiac-enriched lncRNAs in mouse. lncRNAs are also dynamically regulated in failing hearts with mechanical circulatory support, and they are regulated in peripheral blood cells of patients with acute myocardial infarction (MI). In addition, two studies demonstrated that the lncRNAs Fendrr and Braveheart (Bvh) are required for proper development of the heart and body walls and

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cardiovascular lineage commitment, respectively.10,11 Furthermore, bioinformatic analysis of RNA sequencing (RNA-seq) data from both mice and humans revealed that lncRNA expression markedly differs between fetal and adult hearts, and it showed that lncRNA: cis-mRNA gene pairs are involved in heart growth and development.12,13 Although these studies have implicated specific lncRNAs in heart development or failure, how lncRNA expression profiles may be altered during the human fetal-to-adult heart transition and the precise roles of lncRNAs in regulating post-natal myocardial regeneration have not been well characterized.

In this study, we characterized the human transcriptome expression change in the fetal-to-adult heart transition by analyzing publicly available RNA-seq data of human fetal (13th to 17th week of gestation) and normal adult cardiac tissues. Using an array of in vitro and in vivo approaches, we identified an upregulated fetal lncRNA that we called endogenous cardiac regeneration-associated regulator (ECRAR). We showed that ECRAR fostered rat myocardial regeneration in post-natal day 7 and adult rat hearts and attenuated post-infarction adverse remodeling. We further demonstrated that ECRAR was induced by E2F transcription factor 1 (E2F1) and that the downstream mechanism of post-natal myocardial regeneration triggered by ECRAR was through activating extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling. It is thus proposed that ECRAR may represent a promising therapeutic target for CM replacement in heart failure.

RESULTS
Differentially Expressed lncRNAs between Fetal and Adult Hearts
The four RNA-seq datasets of fetal and adult human cardiac tissues generated 189 million clear reads, of which over 170 million (≥81.0%) were uniquely aligned to the human genome (hg19) (Figure S1; Table S1). Among the uniquely mapped reads, 87 million (51.0%) reads mapped to intergenic regions, 69 million (40.5%) reads mapped within exons, and 14 million (8.5%) reads mapped to introns (Figure 1A). The chromosome distribution of these mapped reads in fetal heart was similar to that in the adult heart (Figure S2A). In contrast, the proportions of reads mapped to introns and exons were remarkably different between fetal and adult hearts (Figure S2B). The clear reads were first aligned to the hg19 RefSeq. Reads that failed to be mapped were subsequently mapped to the Ensembl gene set, IncRNA database, and the reference genome, respectively. We identified 152,130 (70.9%) transcripts that were annotated to RefSeq genes, 33,073 (15.4%) were annotated to Ensembl genes, and 28,075 (13.1%) were annotated to NONCODE version (v.)4 genes (Figure 1B; Figure S2C). Compared to the proportion of IncRNAs found in the adult heart, IncRNAs accounted for a lower percentage of total genes in the fetal heart (Figure S2C). Among the 3,958 novel transcripts, 3,830 of the novel transcripts with low coding potential were identified as novel lncRNAs (Figure 1C). The novel and known lncRNAs were shorter and less abundant in length than coding genes (mRNA) (Figures 1D and 1E).

Globally, the cardiac transcriptome of mRNAs and lncRNAs correlated well within groups in the fetal group or adult heart group (Figure S2D). Volcano plots comparing fold change and probability identified 3,211 significantly increased and 578 significantly decreased mRNAs (Figure 1G). By performing hierarchical clustering of differentially expressed protein-coding RNAs, we found that one module with the most significant differentially expressed genes was related to cell cycle function (Figure 1H; Figure S3A). Gene ontology (GO) classification of upregulated genes in fetal hearts showed enrichment for gene categories that regulate the cell cycle, cell cycle phase, cell cycle process, and mitotic cell cycle, whereas downregulated genes were enriched in other functional categories (Figure 1I; Figures S3B and S3C; Table S2). Similarly, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis further revealed that upregulated genes were enriched in proliferation-related pathways, such as the cell cycle (Figure 1I).

We found 3,092 lncRNAs exhibited significant differences (probability >0.8) in expression between fetal and adult hearts, of which 1,343 lncRNAs were upregulated and 1,749 lncRNAs were downregulated in fetal hearts (Figure 1K; Figures S4A and S4B). To explore the potential cis- and trans-regulatory roles of human lncRNAs on protein-coding genes, Pearson correlation coefficients were used to analyze the expression levels of lncRNAs and nearby or distally coding genes. First, to evaluate cis-correlation of expression, we computed interactions between lncRNA-mRNA pairs located within 10 kb upstream and downstream. In total, 54.8% of tested upregulated lncRNA: cis-mRNA gene pairs showed a positive correlation, while 23.1% had a negative correlation (Figure S5A). In contrast, a greater proportion of downregulated lncRNA: cis-mRNA gene pairs were negatively correlated compared to that of those positively correlated (Figure S5B). An example of a cis-acting lncRNA is shown in Figure S5C. GO analysis indicated that both upregulated (r > 0.95) and
downregulated (r > 0.95) IncRNAs: cis-mRNA pairs were associated with the cell cycle and heart development (Figure 1L).

Next, to investigate the trans-correlation of expression, we examined the interactions of IncRNA-mRNA pairs located either at least 1 Mb apart or present on different chromosomes. We found a greater proportion of upregulated IncRNA:trans-mRNA pairs were positively correlated compared to that of those negatively correlated (Figure S5D), whereas downregulated IncRNA:trans-mRNA pairs showed more frequent negative correlations than positive correlations (Figure S5E). GO classification of upregulated IncRNA: trans-mRNA pairs showed enrichment for heart development, whereas downregulated IncRNA:trans-mRNA pairs showed enrichment for other GO functional categories, such as the glucose metabolic process (Figure 1M). These results indicate that the trans-acting IncRNAs are as prevalent as cis-acting IncRNAs in the human heart during the fetal-to-adult transition.

The mRNAs and IncRNAs encoded by mtDNA accounted for a much greater proportion in the adult heart than in the fetal heart (Figures S6A–S6C), which is a finding that indicates that adult hearts have great energy needs. The mitochondrial IncRNA transcript lengths were shorter than those of nonmitochondrial IncRNAs and mRNAs (Figure S6D). The expression of mitochondrial IncRNA transcripts was more abundant than nonmitochondrial IncRNAs and mRNAs, and mitochondrial IncRNAs were less abundant than mitochondrial mRNAs (Figures S6E–S6G). Hierarchical clustering revealed that both mitochondrial mRNAs and IncRNAs in the fetal heart have lower gene expression than those found in the adult heart (Figures S7A and S7B), whereas GO analysis revealed that mitochondrial genes were enriched in energy metabolism (Figure S7C). These findings indicate that the energy demands of the myocardium change from different development stages.

**ECRAR Is Upregulated in the Fetal Heart and Is Potentially Involved in Post-natal Myocardial Regeneration**

From our transcriptome analysis of the human fetal and adult hearts, we identified an IncRNA that was significantly upregulated in the fetal heart more than 12-fold (Figure 2A), and it matched NONCODE: NONHSAG042100.1 (http://www.noncode.org/) and Ensembl: ENST00000523659.5 (http://asia.ensembl.org/index.html?redirect=no). The gene contains four exons, has a poly (A) tail, and is located on chromosome 5 near pituitary tumor-transforming 1 (PTTG1) (Figure 2B). This transcript was an alternative splicing of PTTG1 gene due to retained intron, and it didn’t contain an open reading frame (ORF), which indicated it could not be translated into protein. Coding potential analysis using the Coding-Non-Coding Index tool (CNCI) (https://github.com/www-bioinfo-org/CNCI) indicated that ECRAR was a non-coding RNA (a value of −0.0414; if the value of CNCI < 0, transcripts were considered to be non-coding), which is similar to that of a well-known IncRNA Xist (value of −0.177). In addition, to evaluate whether there exists some possible coding sequence ability of this transcript, we searched the PDB (http://www.rcsb.org/) and blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) databases, and we found no hits by using default parameters.

The enrichment of H3K4me3 and H3K36me3, which are associated with active promoters and active gene bodies, respectively, was observed to be significantly increased in the fetal heart compared to that in the adult heart, which suggests the active chromatin state of ECRAR in the fetal heart (Figure 2B). Conservation analysis revealed that the exons of ECRAR were markedly more conserved than the introns (Figure S8A). By using the BLAST-like alignment tool (BLAT) and Needleman-Wunsch algorithm, we found that the exons were highly conserved across mammalian species, such as chimpanzee, gorilla, pig, rabbit, mouse, and rat, which indicated that this transcript may be a typical non-coding RNA for the mammalian animals (Figure S8B). Rapid amplification of cDNA ends demonstrated that ECRAR was an 851-bp IncRNA (Figures 2C and 2D), which was consistent with the length provided in the Ensembl database (Figure S8C). Using qRT-PCR, we confirmed that ECRAR expression was significantly increased in rat embryonic day (E)12 fetal heart and the expression progressively decreased in rat hearts after birth (Figure 2E). In newborn rat (post-natal day [P]1), we found that ECRAR expression varied among the analyzed tissue types, with expression in the heart and skeletal muscle higher than that found in the brain and liver (Figure 2F). In addition, ECRAR was highly expressed in AC16 cells, while its expression was low in endothelial cells and fibroblasts (Figure 2G).

Findings from the subcellular fractionation assay indicated that ECRAR was mainly located in the cytoplasm (Figure 2H). This
subcellular localization was confirmed by confocal microscopy and fluorescence in situ hybridization (FISH) in rat CMs (Figure 2I). To examine the potential functions of ECRAR, we used coexpression analysis, and we found that ECRAR was associated with several important cell cycle-related genes (Figure 2J). The secondary structure analysis by using by RegRNA Server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi), with default settings, indicated that ECRAR has a stem-loop structure, which may provide the necessary spatial conformation for interactions (Figure S9A). Further, the identified transcripts based on the secondary structure analysis were used to perform the KEGG pathway analysis, which showed that ECRAR may be involved in the Wnt-signaling pathway (Figure S9B). The effect of ECRAR on cardiac hypertrophy was investigated further. Overexpression of ECRAR did not induce the increased secretion of cardiac hypertrophy-related markers, such as hypertrophy-associated molecular marker atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), α-myosin heavy chain (α-MHC), and natriuretic peptides A-like (BNP) (Figure S11A). Immunostaining of ANP confirmed that overexpression of ECRAR did not induce cardiac hypertrophy (Figure S11B). To investigate whether the ECRAR induced the increase in CM volume, we determined the average volume of CMs, and we found no significant increase in the CM volume (Figure 3G). Wheat germ agglutinin (WGA) staining also showed that the cell size of CMs was not increased after transfection with Ad-ECRAR (Figure S11C). We further investigated the effect of ECRAR on cardiac fibrosis. ECRAR overexpression did not increase rat cardiac fibroblast proliferation. We used vimentin, CD90, and Sca-1 to label the fibroblasts. Immunostaining showed overexpression of ECRAR did not induce cardiac fibroblast proliferation (Figure 3H; Figure S12). These findings indicate that ECRAR induces CM proliferation but does not induce CM hypertrophy or increase fibrosis.

ECRAR Overexpression Promoted Rat Post-natal CM Proliferation In Vitro

We first investigated whether our newly discovered ECRAR could stimulate proliferation of post-natal CMs in vitro. Rat ventricular CMs (Figures S10A and S10B) were transfected with Ad-ECRAR PBS for 48 hr (the MOI was 10–20 and the CM transfection efficiency was >95%) (Figure S10C). qRT-PCR confirmed that ECRAR was upregulated in the Ad-ECRAR group (Figure S10D). The CM proliferation was assessed by immunostaining of proliferation markers together with the CM-specific marker α-actinin or cTnT. Overexpression of ECRAR enhanced 5-ethyl-2'-deoxyuridine (EdU) incorporation into CM nuclei, which is a uridine analog incorporated into newly synthesized DNA, suggesting that ECRAR promoted CM proliferation (an increase from 5.7% to 22.7%; Figure 3A). Remarkably, treatment with ECRAR resulted in a significant increase in the number of CMs (Figure 3B).

To further investigate the role of ECRAR on cell mitosis and cytokinesis, transfected cells were assessed by staining for phospho-histone H3 (pH3), a specific marker for mitosis, and for aurora B kinase localization in midbodies, which are transient structures formed during cytokinesis. ECRAR overexpression also resulted in an increased number of pH3-positive CMs (Figure 3C) and aurora B-positive CMs (Figure 3D). Western blot analysis also confirmed that ECRAR induced pH3 and aurora B expression (Figure 3E). To directly visualize newly formed CMs originating from pre-existing CMs, we performed time-lapse imaging of P7 CMs labeled with tetramethylrhodamine ethyl ester (TMRE), a fluorescent mitochondrial dye. We found that the P7 CMs transfected with Ad-ECRAR underwent cell divisions (Video S1; Figure 3F). In contrast, we did not observe any CM division in the P7 CMs transfected with Ad null during the 12-hr time frame (Video S2; Figure 3F).

Figure 3. ECRAR Promotes Rat Post-natal CM Proliferation In Vitro

(A) Representative images and quantification of post-natal day 7 (P7) CMs positive for 5-ethyl-2'-deoxyuridine (EdU) and Ki-67 (*p < 0.05 using t test, n = 6 per group). (B) Quantification of total CM numbers in P7 CMs transfected with Ad-ECRAR or control vectors after 7 days, displayed in (A) (*p < 0.05 using t test, n = 5 per group). (C and D) Representative images and quantification of P7 CMs positive for phospho-histone H3 (pH3) (C) and aurora B (D) (*p < 0.05; n = 5–6 per group). (E) Western blot analysis also confirmed that ECRAR induced pH3 and aurora B expression (*p < 0.05 using t test, n = 5 per group). (F) Representative images taken from a 12-hr time-lapse video of cell division in P7 CMs after transfection with Ad-ECRAR or Ad null. (G) Volume analysis of adult CMs in vitro. L, length; W, width; H, height; n = 3 per group, a total of 200 CMs were analyzed. (H) Representative images of fibroblasts positive for EdU. The cardiac fibroblasts (CFs) were label with CD90 and Sca-1.
identification of CM nuclei in vivo is challenging, we used antibodies against the CM nuclear marker pericentriolar material 1 (PCM-1) to estimate the total numbers of nuclei in the heart (Figure 4H). As previously described, we calculated the CM number, and CMs were significantly increased in adult rats after injection with the Ad-ECRAR (Figure 4I; Figure S15).

**ECRAR Overexpression Promoted Post-MI Cardiomyogenesis and Angiogenesis**

The P7 and adult male Wistar rats that underwent MI surgery were injected with Ad-ECRAR and Ad null in the peri-infarcted area (Figure S16A). Ischemia was judged from both palor of the myocardium and ST-segment elevation on the electrocardiogram (Figure S16B). In the peri-infarcted zone, we found that the overexpression of ECRAR enhanced the number of EdU-positive CMs (Figure 5A), pH3-positive CMs (Figure 5B), and aurora B-positive CMs (Figure S16C) in P7 rat hearts at 14 days post-MI. In adult rat hearts, the intracardiac delivery of Ad-ECRAR also resulted in an increase in the number of EdU-positive CMs (Figure 5C) and pH3-positive CMs (Figure 5D). Because CD31 staining can label endothelial cells and alpha-smooth muscle actin (α-SMA) staining can label vascular smooth muscle, we provided both CD31 and α-SMA staining. In the infarcted zone, the capillary and arteriole densities in the Ad-ECRAR group were significantly higher than those in the Ad-null group (Figures 5E–5H). In the peri-infarcted area, a similar trend in capillary density was observed (Figure 5H).

**ECRAR Overexpression Improved Post-infarction Cardiac Function**

We further determined the effect of ECRAR on cardiac function in adult rats post-MI. The assessment of cardiac function by echocardiography at baseline (before MI) and at 1, 14, 30, and 60 days post-MI showed that the left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were significantly preserved over time in infarcted rats injected with adenovirus-associated virus (AAV)-ECRAR, whereas they progressively declined over time in the AAV-null group (Figures 6A and 6B). In addition, the anterior wall thickness (AWT) of infarcted hearts injected with AAV-ECRAR markedly improved over time (Figures 6A and 6B). No significant enlargements of left ventricular dimensions (LVEDs) and diastolic left ventricular dimensions (LVEDD) were found in hearts injected with AAV-ECRAR at either 30 or 60 days post-MI (Figures 6A and 6B). We found that the myocardial infarct size, based on triphenyltetrazolium chloride staining, was significantly reduced in rats injected with AAV-ECRAR at 60 days post-MI (Figure 6C). Analysis of Masson’s trichrome staining of heart sections also revealed reduced fibrotic scarring in rats injected with AAV-ECRAR at both 14 and 60 days post-MI compared to that in AAV null-injected hearts (Figure 6D). Consistent with our echocardiography and morphometric observations, ECRAR significantly induced myocardial regeneration via CM proliferation after MI, thereby improving post-infarction cardiac function.

**Knockdown of ECRAR Inhibited CM Proliferation and Prevented Post-MI Recovery in Neonatal Hearts**

Next, we investigated whether the knockdown of ECRAR affected CM proliferation and post-MI cardiac remodeling. 1-day-old rat ventricular CMs were transfected with short hairpin RNA (sh)-ECRAR and negative control shRNA (sh-NC). qRT-PCR confirmed that ECRAR was downregulated in the sh-ECRAR group (Figure S16D). The knockdown of ECRAR resulted in a significant decrease in the percentage of EdU-positive CMs (Figure 7A), pH3-positive CMs (Figure 7B), and aurora B-positive CMs (Figure 7C). Immunofluorescence analysis showed a marked decrease in the number of EdU-positive CMs (Figure 7D) and pH3-positive CMs (Figure 7E) in those rats injected with sh-ECRAR as compared with those injected with sh-NC. Decreased ECRAR levels inhibited functional post-MI recovery in P1 rat hearts, whereas no effect was observed with the delivery of sh-NC (Figure 7F). We further assessed the effect of ECRAR on human AC16 cells. In vitro administration of sh-ECRAR caused a significant decrease in AC16 CM proliferation, as shown by immunostaining for markers of pH3 and aurora B (Figure 7G).

**ECRAR Is Upregulated through E2F1 Binding at the Promoter Region**

We then aimed to identify the transcription factors responsible for ECRAR expression. To promote the binding sites in the promoter region of ECRAR, we used the Jaspar database (http://jaspar.binf.ku.dk/) with default settings, and we predicted that E2F1 might bind to the promoter region of ECRAR (−855 to −866 bp relative to the transcription start site [TSS]) (Figure S17A). Based on our coexpression analysis of ECRAR, we found comparable expression of ECRAR and E2F1 in human fetal and adult hearts (Figure S17B). The AC16 human CMs transfected with E2F1-containing vector increased ECRAR expression as determined by qRT-PCR (Figure S17C). Chromatin immunoprecipitation (ChIP)-qPCR with an anti-E2F1 antibody further confirmed that E2F1 binds to the ECRAR promoter region in CMs (Figures S17D and S17E), which indicates that E2F1 is involved in ECRAR transcription. ECRAR promoter regions with or without an E2F1 motif mutation were cloned into pGL3-basic reporter plasmids (Figure S17F, left). We found that luciferase expression was significantly reduced when the putative E2F1-binding site

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**Figure 4. ECRAR Promotes Rat Post-natal CM Proliferation In Vivo**

(A) Immunostaining and quantification of EdU-positive CMs in P7 rat in vivo (*p < 0.05 using t test; n = 5–6 per group), (B) Immunostaining and quantification of pH3-positive CMs in P7 rat in vivo (*p < 0.05 using t test; n = 5–6 per group), (C) Immunostaining and quantification of aurora B-positive CMs in P7 rat in vivo (*p < 0.05 using t test; n = 5–6 per group), (D and E) Immunostaining (D) and quantification (E) of EdU-positive CMs in adult rat in vivo (*p < 0.05 using t test; n = 5 per group), (F and G) Immunostaining (F) and quantification (G) of pH3-positive CMs in adult rat in vivo (*p < 0.05 using t test; n = 5 per group), (H) The labeling strategy used to identify CM nuclei and the number of nuclei per CM. CM nuclei were labeled with antibodies against PCM-1 (red), and the cell borders were labeled with antibodies against WGA (green). (I) Serial section analysis revealed that the number of CMs increased in the adult rat hearts after injection with Ad-ECRAR as compared to injection with Ad null.
Figure 5. ECRAR Induces Myocardial Regeneration and Promotes Angiogenesis after MI

(A) Immunostaining and quantification of EdU-positive CMs in P7 rats post-MI in peri-infarct zones (*p < 0.05 using t test; n = 5–6 per group). (B) Immunostaining and quantification of pH3-positive CMs in P7 rats post-MI in peri-infarct zones (*p < 0.05 using t test; n = 5 per group). (C) Immunostaining and quantification of EdU-positive CMs in adult rats post-MI in peri-infarct zones (*p < 0.05 using t test; n = 5–6 per group). (D) Immunostaining and quantification of pH3-positive CMs in adult rats post-MI in peri-infarct zones (*p < 0.05 using t test; n = 5 per group). (E) Representative immunostaining images and quantification of the capillaries by staining with anti-rat CD31 antibody in adult Ad-ECRAR and Ad-null hearts.*p < 0.05 using Student’s t-test. (legend continued on next page)
was mutated (Figure S17F, right). These findings demonstrate that the expression of ECRAR is transcriptionally regulated by E2F1.

**ECRAR Promoted CM Proliferation via an ERK1/2-Dependent Mechanism**

We performed microarray analysis of gene expression profiles in CMs, and we found that ECRAR overexpression resulted in significant changes in gene expression (1,568 coding genes changed >1.5-fold, all p < 0.05; Figure S18A), with upregulation of many cell proliferation and cell cycle-related genes (Figure 8A; Figure S18B). GO enrichment and KEGG pathway analysis of upregulated genes indicated that the cell cycle pathway was a principal pathway associated with the overexpression of ECRAR (Figures S18C and S18D). These findings suggest ECRAR has a prominent role mediating regulation of the cell cycle during CM proliferation.

We next sought to determine the underlying molecular mechanism of how ECRAR regulates CM proliferation. Because overexpression or
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A

Hoechst  α-actinin  EdU  Merge  Zoom

P1: sh-ECRAR  P1: sh-ECRAR

P1: sh-NC  P1: sh-NC

50μm  50μm

Edu + CMs in vitro (%)  *

B

Hoechst/α-actinin/pH3

P1: sh-ECRAR  P1: sh-ECRAR

P1: sh-NC  P1: sh-NC

100μm  100μm

pH3 + Cms in vitro (%)  *

C

P1:Sh-ctrl  P1:sh-NC

P1:sh-ECRAR  P1:sh-NC

Hoechst/α-actinin/Aurora B

Aurora B + Cms in vitro (%)  *

D

Hoechst/α-actinin/EdU

P1:sh-ECRAR  P1:sh-ECRAR

P1:sh-NC  P1:sh-NC

40μm  40μm

EdU + Cms in vivo (%)  *

E

Hoechst/α-actinin/pH3

P1:sh-ECRAR  P1:sh-NC

P1:sh-ECRAR  P1:sh-NC

40μm  40μm

pH3 + Cms in vivo (%)  *

F

P1: sh-ECRAR  P1: sh-NC

500μm  500μm

Fibrosis area (%)  *

G

Hoechst/α-actinin/Aurora B  Hoechst/α-actinin/pH3

AC16: sh-ECRAR  AC16: NC

20μm  20μm

(legend on next page)
knockdown of ECRAR had no effect on the expression of its nearby coding gene PTTG1 (Figures S19A and S19B), we excluded the possibility that ECRAR acts by influencing its nearby gene of PTTG1 in cis. To search for potential ECRAR-interacting proteins, we performed pull-down assays using biotinylated ECRAR followed by mass spectrometry (MS). By screening the MS data, we found three possible related proteins might be involved in the proliferation (Figures S20 and S21). Among them, the mitogen-activated protein kinase 3 (MAPK3), also known as ERK1/2, which has been shown to play a key role in regulating cell proliferation and cell cycle progression (Figure S20; Figure 8B). Prediction of the ECRAR structure indicated that the nucleotide position of 151–202 interacted with ERK1/2 (Figures S22A and S22B). In addition, the binding sites are conserved between human and rats (Figure S22C). Immunoblotting further confirmed the identification of ERK1/2 (Figure 8C). We used RNA-binding immunoprecipitation (RIP) to further verify the interaction between ECRAR and ERK1/2 (Figure 8D). RNA FISH and immunofluorescence also showed ECRAR colocalized with ERK1/2 in the cytoplasm (Figure 8E).

The phosphorylation of ERK1/2 is essential for ERK-mediated G1/S transition. During this process, the ERK1/2 translocates into the nucleus and activates the transcription of many genes involved in cell proliferation. We first investigated whether the binding to ECRAR affects ERK1/2 phosphorylation status. As expected, western blot analysis revealed that phosphorylation of ERK1/2 was increased with ECRAR overexpression in P7 CMs (Figure 8F). In addition, overexpression of ECRAR in CMs triggered ERK1/2 translocation into the nucleus (Figure 8G).

The inhibition of ERK1/2 counteracted the decrease in the percentage of G1/G0 phase cells by ECRAR overexpression (Figure 8H). ERK1/2 inhibition also ablated ECRAR-induced CM proliferation (Figure 8I). We further found that activation of ERK1/2 induced by Inc-ECRAR led to the elevated expression of cyclin D1, cyclin E1, and E2F1 proteins, and this elevation could be ablated by using ERK1/2 inhibition (Figure 8I). Overexpression of ECRAR markedly increased the E2F1 expression, whereas both cyclin D1 inhibition and cyclin E1 inhibition could partly counteract the increase (Figures 8K and 8L).

Thus, ECRAR promoted proliferation in post-natal CMs, at least in part, via an ERK1/2-dependent mechanism. The above results, together with the upstream mechanism that ECRAR is transcriptionally upregulated by E2F1, implicate the existence of a positive feedback loop between E2F1 and ECRAR (Figure S23).

DISCUSSION

In this study, we systematically characterized human long non-coding transcriptome changes during the human fetal-to-adult heart transition, and we identified a novel human-derived ECRAR and demonstrated that it promotes post-natal CM proliferation and induces post-MI functional recovery via the ERK1/2-dependent mechanism. Moreover, the E2F1–ECRAR–ERK1/2 pathway forms a positive feedback loop, which provides sustained activation of the ERK1/2 to drive cell cycle reentry (Figure S23). These findings indicated that ECRAR may be a valuable therapeutic target for inducing cardiac regeneration.

Comprehensive analysis of transcriptome changes during the human fetal-to-adult heart transition may reveal novel gene targets that reactivate fetal genes and retain a fetal pattern of regenerative capacity. In this study, we provided a global analysis of mRNA and IncRNA profiles during the human fetal-to-adult heart transition, and we identified over 1,000 differentially expressed IncRNAs. The marked differences in the expression pattern and abundance of these mRNAs and IncRNAs implicated their distinct biological roles in myocardial regeneration, an interpretation supported by the finding that functional annotation of upregulated mRNAs in the fetal heart was enriched in the cell cycle or mitotic cell cycle pathways. In addition, both differentially expressed IncRNA: cis-mRNA gene pairs and differentially expressed IncRNA: trans-mRNA gene pairs were enriched in the cell cycle- or heart development-related functional categories. In contrast to recent findings that only cis-regulation is a major regulatory mechanism of cardiac IncRNAs, our study suggests that trans-acting IncRNAs are as prevalent as cis-acting IncRNAs in the human heart during the fetal-to-adult transition.12,13 Additionally, we inferred from coexpression network analysis the putative function of differentially expressed IncRNAs, and we found that these IncRNAs may be involved in the role of heart development and the cell cycle. Thus, we proposed that the differentially expressed IncRNAs may be involved in reactivation of the fetal genetic program in the adult heart.

A particularly striking finding in our study was the demonstration that our newly identified ECRAR significantly promoted post-natal CM proliferation, without induced CM hypertrophy or fibroblast proliferation, and attenuated post-MI remodeling. As a field, we have believed for decades that the human adult heart lost its capacity for self-renewal, while new evidence has shown that CMs may renew themselves, albeit at a slow rate (approximately 1% per year at the age of 20 and 0.3% at the age of 75).14 These studies suggest that stimulating endogenous cardiac regeneration may be a viable and promising therapeutic approach. Many researchers have attempted to reactivate adult CM proliferation either by overexpressing cell cycle activators, such as cyclin A2 and cyclin D2, or by removing inhibitors, such as cyclin-dependent kinase (CDK) inhibitors, in the adult heart.15–19 Other investigators extended the role of microRNAs
In the current study, we not only advanced the current understanding of lncRNA biology in post-natal CM proliferation and post-natal regeneration but also showed a new strategy in that modulation of lncRNA in adult CMs may have a therapeutic benefit. In contrast to CM regeneration, we also found that the overexpression of ECRAR did not cause cardiac hypertrophy or myocardial fibrosis, which indicated that ECRAR might be an ideal gene target for future research. We observed that ECRAR overexpression induced an increase in vascular density. It is likely the paracrine mechanisms augment angiogenesis activity. Previous studies have shown that the CM influences endothelium response via paracrine factors, such as angiopeptins (vascular endothelial growth factor [VEGF]), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF), which efficiently foster angiogenesis. Importantly, the transient expression of ECRAR in the adult heart of rats after MI reduced infarct scarring and improved cardiac function. Our study provides important insights into the role of lncRNA ECRAR in cardiac regeneration and cardiac function, and it presents a convincing rationale for the potential therapeutic benefit of ECRAR activation in post-natal CMs.

We found that the phosphorylation of ERK1/2 was induced by Ad-ECRAR along with the process of cytoplasmic-to-nuclear translocation. When ERK1/2 translocates into the nucleus, it can activate transcription of many genes involved in cell proliferation. We further demonstrated that ECRAR was induced by transcription factor E2F1 and that the downstream mechanism of post-natal myocardial regeneration triggered by ECRAR was through ERK1/2 signaling. The withdrawal of mammalian CMs from the cell cycle soon after birth is believed to be the primary mechanism for the loss of functional cardiac regenerative capacity. Therefore, reactivating CM proliferation through cell reentry is particularly appealing. Previous studies have demonstrated that the genetic manipulation of cell cycle-regulatory factors, such as cyclin D1/D2 and cyclin A2, could induce increased CM-cycling activity and evoke a regenerative response. ERK1/2 signaling has been demonstrated to play a central role in cell cycle control. Here, we showed that ECRAR could promote CM proliferation by interacting with ERK1/2 and further activating ERK1/2 kinase activity by phosphorylation. ERK1/2 activation plays a fundamental role in the G1/S transition, and it is required for the induction of positive regulators of the cell cycle. We found that the activation of ERK1/2 by ECRAR led to the induction of cell cycle activators of cyclin D1, cyclin E1, and E2F1 proteins. The activation of ERK1/2 has been reported to induce cyclin D1 protein expression through many mechanisms. The newly synthesized cyclin D1 interacts with existing CDK4/6 (cyclin-dependent kinases) and forms an active cyclin D1-CDK4/6 complex, which plays a critical role in the G1/S transition. In addition, cyclin E is expressed in the later G1 phase and together with Cdk2 to form an active cyclin E1-Cdk2 complex, which also promotes the cell cycle entry into the S phase. Moreover, the cyclin D1-CDK4/6 complex and cyclin E1-Cdk2 complex are known to enforce the phosphorylation of the retinoblastoma protein (pRb), resulting in full activation of E2F1.

The activation of E2F1 further increased the ECRAR transcription in the ECRAR promoter. Sustained activation of ERK1/2 is necessary for successful G1- to S-phase progression. We identified that E2F1-ECRAR-ERK1/2 signaling formed a positive feedback loop, which helps contribute to the irreversibility of the G1/S transition, resulting in cell cycle reentry and induction of CM proliferation.

In conclusion, differentially expressed lncRNAs during the human fetal-to-adult heart transition may be involved in reactivation of the fetal genetic program in the adult heart, and our newly discovered human-derived ECRAR promoted post-natal myocardial regeneration and attenuated aberrant post-infarction remodeling via ERK1/2 signaling. These findings suggest that ECRAR may serve as a novel effective gene target for future therapeutic strategies in heart failure.

MATERIALS AND METHODS

Animals
All experiments in this study were performed in accordance with Southern Medical University Institutional Guidelines for Animal Research, and the investigation conformed to the NIH’s Guide for the Care and Use of Laboratory Animals. For additional details regarding the animals and methods used in our study, please refer to the Supplemental Materials and Methods.
Transfection of AdV or Adeno-Associated Virus 9 Vectors
In in vitro experiments, the AdV or AAV9-containing GFP vectors for depletion or overexpression of ECRAR were added to cells with an MOI of 10. A scramble vector and PBS were used as controls. After 24 hr, cells were maintained in normal fresh medium for 48 hr before proceeding with experiments and analysis. After transfection, cells were used for ECRAR overexpression experiments.

In P1 and P7 neonatal rats, the AdV or AAV9-containing GFP vectors for depletion or overexpression of ECRAR and empty control vector were injected into the heart ventricles of P1 and P7 rats at 3 sites, at a dose of $3 \times 10^8$ viral genome particles per animal, using an insulin syringe with an incorporated 30G needle. In adult rats, the viral genome particles were injected into left ventricles at a dose of $3 \times 10^{11}$ viral genome particles per animal. Ad-GFP was used to determine the distribution of AdV-mediated ECRAR expression in the myocardium. Rats received an intramyocardial injection of Ad-ECRAR and Ad null as described above. 14 days following injection, the rats were captured to examine GFP fluorescence with a Bruker In-Vivo FX Pro system (Bruker, MA, USA).

Establishment of the MI Model
MI was induced by ligation of the left anterior descending coronary artery as previously described. P1 and P7 rats were anesthetized by cooling on an ice bed for 4 min, whereas adult rats were intraperitoneally anesthetized with 3% pentobarbital sodium (40 mg/kg) following tracheal intubation for artificial ventilation. The left coronary artery (LAD) was ligated with silk suture 2 mm distal from the ascending aorta. Ischemia was judged from both pallor of the myocardium and ST-segment elevation on the electrocardiogram (Figure S16A). Immediately after LAD ligation, Ad-ECRAR (or AAV-ECRAR) or Ad null (or AAV null) was injected into the myocardium bordering the infarct zone (single injection), using an insulin syringe with a 30G needle (Figure S16B). The chest was closed and warmed for several minutes until recovery. EdU was administered intraperitoneally every 2 days for a total period of 10 days.

Computational Methods and Statistical Analysis
All computational procedures were carried out using in-house programs written in R or analyzed in Excel 2007 (Microsoft, Redmond, WA, USA) and Stata 12.0 (Stata, College Station, TX, USA). Quantitative data are expressed as mean ± SD. Differences between two groups were evaluated using unpaired Student’s t tests, and one-way ANOVA with post hoc analysis using Bonferroni tests were used for multiple comparisons with SPSS 16.0 (SPSS, Chicago, IL, USA). For all tests, p < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes twenty three figures, six tables, Supplemental Materials and Methods, and two videos and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.10.021.

AUTHOR CONTRIBUTIONS
Y.C. and J.B. conceived the study. Y.C., X.L., and B.I. performed most of the experiments. Y.C. and H.W. performed the bioinformatic analyses. M.L., S.H., Y.S., G.C., X.S., and C.H. helped with various experiments and statistical analyses. Y.L., W.L., and J.B. supervised the study. Y.C. and J.B. wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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Supplemental Information

Long Non-coding RNA ECRAR Triggers
Post-natal Myocardial Regeneration by Activating
ERK1/2 Signaling

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Figure S1. Workflow for the comprehensive quantification of the transcriptome

Workflow to comprehensively quantify mRNAs and long non-coding RNAs (lncRNAs) in fetal and adult heart. RNA sequencing (RNA-seq) reads underwent transcriptome reconstruction and in silicopredictive pipelines to identify novel transcripts; 3830 novel lncRNAs were identified by RNA-seq, of which 236 were identified as novel multi-exon lncRNAs. A compiled lncRNA annotation database, which included NONCODE4.0, was generated and used for quantitative analyses of these lncRNAs. The RefSeq and Ensemble databases were used for mRNA quantification, and the RNA-seq results underwent differential expression, feature, and functional analysis as well as coexpression network analyses. RPKM indicates reads per kilobase of exon per million mapped reads.
Figure S2: The transcriptional signature of genes in fetal and adult heart.

(A) Circos plot showing reads mapped to individual chromosomes in fetal and adult heart, respectively. The inner track shows reads mapped to the sense and antisense strand and the outer track indicates reads mapped to both strands together. (B) Pie charts showing read count distributions of exons, introns, and intergenic regions in fetal and adult heart, respectively. (C) Pie chart showing composition of RefGene mRNAs, Ensembl mRNAs, known long non-coding RNAs (lncRNAs), and novel lncRNAs. (D) Transcriptome correlations between expressed mRNAs and lncRNAs derived from fetal and adult heart samples, respectively.
Figure S3: Differential expression of mRNAs between fetal and adult heart.

(A) Comparison of cell cycle related mRNA fold changes by RNA-seq and qRT-PCR. (B) Gene Ontology (GO) analysis of biological processes, cellular components, and molecular functions associated with upregulated mRNAs in fetal heart compared to adult heart. (C) Gene Ontology (GO) analysis of biological processes, cellular components, and molecular functions associated with downregulated mRNAs in fetal heart compared to adult heart.
Figure S4: Validation of differential expression of long non-coding RNAs (lncRNAs) between fetal and adult heart.

(A) Heat maps showing unsupervised hierarchical clustering of all lncRNA transcriptomes. (B) Comparison of lncRNA fold changes [(log2 ratio (fetal heart/ adult heart))] by RNA-seq and qRT-PCR.
Figure S5: Functional analysis of long non-coding RNAs (lncRNAs) in fetal and adult heart.
(A) Histograms of Pearson correlations between up-regulated lncRNA and their cis-mRNA gene pairs. (B) Histograms of Pearson correlations between down-regulated lncRNAs and their cis-mRNA gene pairs. (C) An example of cis-acting lncRNA. (D) Histograms of Pearson correlations between up-regulated lncRNA and their trans-mRNA gene pairs. (E) Histograms of Pearson correlations between down-regulated lncRNAs and their trans-mRNA gene pairs.
Figure S6: Transcriptional signature of mitochondrial mRNAs and lncRNAs in the fetal and adult heart.

(A) Pie charts showing read count distributions of mitochondrial and non-mitochondrial mRNAs and lncRNAs in human heart as percentages of the total read counts. (B and C) Pie charts showing read count distributions of mitochondrial and non-mitochondrial mRNAs and lncRNAs in fetal hearts or adult hearts. Kernel density plot of the transcript length of mitochondrial and non-mitochondrial mRNAs and lncRNAs. (D) Transcript abundance of mitochondrial and non-mitochondrial mRNAs and lncRNAs in fetal hearts. (E) Transcript abundance of mitochondrial and non-mitochondrial mRNAs in adult heart. (F) Transcript length of mitochondrial and non-mitochondrial lncRNAs in fetal heart. (G) Transcript length of mitochondrial and non-mitochondrial lncRNAs in adult heart.
Figure S7: differentially expressed mitochondrial mRNAs and lncRNAs between fetal and adult heart

(A) Heat maps show unsupervised hierarchical clustering of all mitochondrial mRNAs. (B) Heat maps show unsupervised hierarchical clustering of all mitochondrial lncRNAs. (C) Gene ontology (GO) analysis of biological processes associated with mitochondrial mRNAs in fetal and adult heart.
Figure S8: Analysis of ECRAR features.

(A) Conservation analysis of ECRAR in different mammalian species. (B). Conservation analysis of ECRAR revealed that the exons of ECRAR were markedly more conserved than the introns. (C) The sequence of ECRAR provided by NONCODE and Ensemble database (ID:ENST00000523659.5).
**Figure S9: Functional analysis of ECRAR.**

(A) Prediction of lncRNA-ECRAR structure based on minimum free energy (MFE) and partition function (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi/). Color scale indicates the confidence for the prediction for each base with shades of red indicating strong confidence. **(B)** KEGG pathway analysis of genes may interact with ECRAR based on the motif analysis.
**Fig. S10: Overexpression of ECRAR.**

(A and B) Representative images and quantification of isolated cardiomyocyte, the purity of isolated cardiomyocyte is more than 90%.. (C). CMs were transfected by Ad-ECRAR at different multiple of infection (MOI) (n=3 each groups). Scale bar: 100μm. (D) Infection efficiency of CMs assayed by qRT-PCR. Data are shown as mean ± s.e.m. *P < 0.05 vs Ad-null.
Figure S11: The effect of ECRAR on cardiac hypertrophy.

(A). Cardiac hypertrophy related markers such as β-myosin heavy chain(β-MHC), α- myosin and heavy chain(α-MHC) and natriuretic peptides A-like (BNP) was assayed by quantitative RT-PCR (qRT-PCR) in rat hearts after transfected with Ad-ECRAR or Ad-null. (B). Representative images and quantification of cardiomyocytes (CMs) positive for atrial natriuretic peptide (ANP). n = 4 per group (C). CM sizes of left ventricle heart sections are evaluation by wheat germ agglutinin (WGA) staining of P7 and adult rat hearts injected with Ad-ECRAR or Ad-null (CMs sizes pooled from the analysis of 5 rats).
Figure S12 The effect of ECRAR on cardiac fibrosis.

(A) Representative images and quantification of cardiac fibroblasts (CFs) positive for ki-67. n = 4 per group. The cardiac fibroblasts (CFs) were labeled with vimentin. (B) Quantification of fibroblasts positive for EdU displayed in figure 3H. n = 4 per group. The cardiac fibroblasts (CFs) were labeled with CD90 and Sca-1.
**Figure S13: Overexpression of ECRAR in vivo.**

(A) Representative images of the same rats showing the distribution of Ad-ECRAR expressing the GFP as measured by in vivo bioluminescent imaging in adult rats that received an intramyocardial injection of Ad-ECRAR after 14 days. The arrow in the image of biominescent indicates heart tissues. Schematic of the MI experiments in adult rat. (B) Infection efficiency of hearts assayed by qRT-PCR after transfected with Ad-ECRAR or Ad-null (*P < 0.05 vs Ad-null. n = 4 per group).
Figure S14: ECRAR Overexpression Promoted Rat Postnatal CM Proliferation in vivo.
(A and B) Representative images and quantification of CMs positive for Ki-67 in rats injected with Ad-ECRAR or Ad-null. n = 4 per group. *P < 0.05 vs Ad-null. Scale bar: 50μm. (C) Representative images and quantification of P7 rat hearts and the ratio between heart and body weight (*P < 0.05; Ad-ECRAR vs. Ad-null group; n=4 per group). (D) Representative images and quantification of adult rat hearts and the ratio between heart and body weight (*P < 0.05; Ad-ECRAR vs. Ad-null group; n=4 per group).
Fig. S15  Sections 8–18 out of 25 serial sections are shown.

The sections are stained with antibodies against WGA to visualize the cell borders. The cTnT was used to identify CMs. A binuclear myocyte is followed through all sections.
Figure S16: OverECRAR induces heart regeneration and improved cardiac remodeling after myocardial infarction (MI).

(A) Schematic of the MI experiments in adult rat. (B) Elevation of ST segment showed by ECG in MI model. (C) Representative images and quantification of CMs positive for aurora B in P7 rats post-MI (*P<0.05 using t-test; n= 5-6 per group). (D) The lnc-ECRAR expression in CMs after tranfected with sh-ECRAR or sh-CN. (*P<0.05, n=5 per group)
Figure S17. ECRAR expression is regulated through E2F1 binding at the promoter region. (A) Predicted binding sequence in the ECRAR promoter region. (B) ECRAR and E2F1 expression in human fetal and adult hearts. (C) ECRAR expression assayed by qRT-PCR in AC16 human cardiomyocytes (CMs) transfected with either E2F1 or control vector. (D) Chromatin immunoprecipitation (ChIP)-qPCR using anti-E2F1 or anti-IgG antibodies was performed to assess binding between ECRAR and E2F1 (*P<0.05 using t-test, n=5 per group). (E) Image of agarose electrophoresis showing Chromatin immunoprecipitation (ChIP)-qPCR using anti-E2F1 or anti-IgG antibodies was performed to assess binding between ECRAR and E2F1. (F) Binding of ECRAR and E2F1 as confirmed by luciferase reporter assay. Diagram showing ECRAR promoter region or deletion variants with (Mut) or without (Wt) mutations in the E2F1 binding site cloned upstream of firefly luciferase. Luciferase activity was measured in AC16 human CMs cotransfected with either the E2F1 expression vector or an empty vector (*P<0.05 using t-test, n=5 per group).
Figure S18: Microarray analysis of gene expression profiles in CMs overexpressing ECRAR.

(A) Volcano plot of differentially expressed genes from transcriptome microarray analysis in cardiomyocytes (CMs) transfected with Ad-ECRAR or control. (B) Comparison of cell cycle related gene fold changes by microarray analysis and qRT-PCR. (C) Gene Ontology (GO) enrichment analysis of up-regulated (red) and down-regulated (green) mRNAs; (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms (Y-axis) for up-regulated (red) and down-regulated (green) mRNAs.
Figure S19: Microarray analysis of gene expression profiles in CMs overexpressing ECRAR.

(A) qPCR detection of ECRAR and its nearby coding gene PTTG1 levels when the ECRAR overexpression (*P<0.05 vs Ad-null using t-test). (B) qPCR detection of ECRAR and its nearby coding gene PTTG1 levels when the ECRAR knockdown (*P<0.05 vs Ad-null using t-test).
Figure S20: Supporting peptides of ERK1/2 provided by MS.
(A-B) Two peptides of ERK1/2 detected by MS are indicated above the graphs.
Figure S21: Supporting peptides of provided by MS.
(A) One peptides of Proliferation-associated protein 2G4 (PA2G4) detected by MS are indicated above the graphs. (B) One peptides of Heat shock cognate 71 kDa protein (HSP7C) detected by MS are indicated above the graphs.
Figure S22: Prediction of lnc-ECRAR and ERK1/2 protein interactions

(A) catRAPID omics predicted the possible binding sites of lncRNA-ECRAR and ERK1/2, positive values indicate possible binding sites. (B) catRAPID omics predicted the that the lnc-ECRAR nucleotide position of 151 to 202 interacted with ERK1/2. (C) The binding sites of lnc-ECRAR and ERK1/2 was located in the third exons and was conserved between humans and rats.
Figure S23: Schematic model illustrating ECRAR-mediated myocardial regeneration.
### Table S1: Summary of RNA-sequencing reads

|               | Fetal_1     | Fetal_2     | Adult_1     | Adult_2     |
|---------------|-------------|-------------|-------------|-------------|
| Total reads   | 74678048    | 52457369    | 31449650    | 30835782    |
| Mapped Reads  | 93.0%       | 91.2%       | 89.7%       | 89.7%       |
| Unique match  | 82.4%       | 81.0%       | 83.9%       | 83.8%       |
### Table S2: Cell cycle related gene list of differentially expressed mRNAs between fetal and adult hearts

| gene   | symbol | Name                                                        | log2Ratio(B/A) | Probability |
|--------|--------|-------------------------------------------------------------|----------------|-------------|
| 64682  | ANAPC1 | anaphase promoting complex subunit 1(ANAPC1)               | -1.754520212   | 0.840597    |
| 51529  | ANAPC11| anaphase promoting complex subunit 11(ANAPC11)             | -2.191767572   | 0.872986    |
| 25906  | ANAPC15| anaphase promoting complex subunit 15(ANAPC15)             | -2.005412472   | 0.853881    |
| 29945  | ANAPC4 | anaphase promoting complex subunit 4(ANAPC4)               | -3.300430248   | 0.908959    |
| 51434  | ANAPC7 | anaphase promoting complex subunit 7(ANAPC7)               | -1.489688048   | 0.823118    |
| 9181   | ARHGEF2| Rho/Rac guanine nucleotide exchange factor 2(ARHGEF2)     | -1.487321218   | 0.824735    |
| 55726  | ASUN   | asunder, spermatogenesis regulator(ASUN)                   | -2.568597043   | 0.891408    |
| 6790   | AURKA  | aurora kinase A(AURKA)                                     | -3.040706379   | 0.914411    |
| 9564   | BCAR1  | BCAR1, Cas family scaffolding protein(BCAR1)               | -1.567373197   | 0.834282    |
| 332    | BIRC5  | baculoviral IAP repeat containing 5(BIRC5)                 | -7.443659659    | 0.991862    |
| 57448  | BIRC6  | baculoviral IAP repeat containing 6(BIRC6)                 | -1.568222488   | 0.828857    |
| 79866  | BORA   | bora, aurora kinase A activator(BORA)                      | -3.040782608    | 0.876174    |
| 699    | BUB1   | BUB1 mitotic checkpoint serine/threonine kinase(BUB1)     | -7.138976627    | 0.996802    |
| 701    | BUB1B  | BUB1 mitotic checkpoint serine/threonine kinase B(BUB1B)  | -7.07903303     | 0.94974    |
| 9184   | BUB3   | BUB3, mitotic checkpoint protein(BUB3)                     | -1.889967604    | 0.859764    |
| 8900   | CCNA1  | cyclin A1(CCNA1)                                           | -9.009763513    | 0.981569    |
| 890    | CCNA2  | cyclin A2(CCNA2)                                           | -6.814914257    | 0.996892    |
| 891    | CCNB1  | cyclin B1(CCNB1)                                           | -5.68880258     | 0.989204    |
| 9133   | CCNB2  | cyclin B2(CCNB2)                                           | -11.26455697    | 0.997252    |
| 894    | CCND2  | cyclin D2(CCND2)                                           | -1.942922218    | 0.863348    |
| 9134   | CCNE2  | cyclin E2(CCNE2)                                           | -4.36933404     | 0.948991    |
| 899    | CCNF   | cyclin F(CCNF)                                             | -3.291656441    | 0.902761    |
| 900    | CCNG1  | cyclin G1(CCNG1)                                           | -1.667437362    | 0.847531    |
| 901    | CCNG2  | cyclin G2(CCNG2)                                           | -1.44443743     | 0.803304    |
| 904    | CCNT1  | cyclin T1(CCNT1)                                           | -1.51025585     | 0.817702    |
| 905    | CCNT2  | cyclin T2(CCNT2)                                           | -1.578541563    | 0.834309    |
| 219771 | CCNY   | cyclin Y(CCNY)                                             | -1.316297113    | 0.810777    |
| 23607  | CD2AP  | CD2 associated protein(CD2AP)                              | -1.745083158    | 0.842402    |
| 8872   | CDC123 | cell division cycle 123(CDC123)                            | -1.452279869    | 0.818636    |
| 8881   | CDC16  | cell division cycle 16(CDC16)                              | -1.396216138    | 0.816444    |
| 991    | CDC20  | cell division cycle 20(CDC20)                              | -7.015372788    | 0.995563    |
| 8697   | CDC23  | cell division cycle 23(CDC23)                              | -1.935691867    | 0.859297    |
| 993    | CDC25A | cell division cycle 25A(CDC25A)                            | -4.541029083    | 0.954991    |
| 994    | cdc25b | cell division cycle 25B(CDC25B)                            | -2.651690967    | 0.900884    |
| Gene Code | Gene Description                  | Value   | p-value  |
|-----------|-----------------------------------|---------|----------|
| CDC25C    | cell division cycle 25C(CDC25C)   | -6.8888| 0.969524 |
| CDC27     | cell division cycle 27(CDC27)     | -1.3063| 0.080591 |
| CDC42     | cell division cycle 42(CDC42)     | -1.5492| 0.836456 |
| CDC6      | cell division cycle 6(CDC6)       | -5.6310| 0.983985 |
| CDC7      | cell division cycle 7(CDC7)       | -5.5199| 0.981767 |
| CDC2A     | cell division cycle associated 2(CDCA2) | -4.8266| 0.965644 |
| CDCA4     | cell division cycle associated 4(CDCA4) | -2.1742| 0.859243 |
| CDCA5     | cell division cycle associated 5(CDCA5) | -4.7718| 0.965761 |
| CDCA7     | cell division cycle associated 7(CDCA7) | -5.8967| 0.983985 |
| CDCA7L    | cell division cycle associated 7 like(CDCA7L) | -1.7637| 0.851456 |
| CDK1      | cyclin dependent kinase 1(CDK1)   | -7.4730| 0.998536 |
| CDK13     | cyclin dependent kinase 13(CDK13) | -1.2969| 0.800223 |
| CDK14     | cyclin dependent kinase 14(CDK14) | -1.7327| 0.849678 |
| CDK19     | cyclin dependent kinase 19(CDK19) | -2.2665| 0.876165 |
| CDK2      | cyclin dependent kinase 2(CDK2)   | -2.4511| 0.889315 |
| CDK5      | cyclin dependent kinase 5(CDK5)   | -2.3131| 0.843947 |
| CDK6      | cyclin dependent kinase 6(CDK6)   | -4.5024| 0.965294 |
| CDK7      | cyclin dependent kinase 7(CDK7)   | -1.7279| 0.80448 |
| CKS2      | CDC28 protein kinase regulatory subunit 2(CKS2) | -5.3026| 0.983285 |
| HAUS1     | HAUS augmin like complex subunit 1(HAUS1) | -2.9468| 0.907557 |
| HAUS3     | HAUS augmin like complex subunit 3(HAUS3) | -1.6254| 0.824115 |
| HAUS8     | HAUS augmin like complex subunit 8(HAUS8) | -3.0112| 0.891219 |
| KATNA1    | katanin catalytic subunit A1(KATNA1) | -1.5613| 0.832908 |
| KIF11     | kinesin family member 11(KIF11)   | -7.4893| 0.996775 |
| KIF14     | kinesin family member 14(KIF14)   | -8.0838| 0.987776 |
| KIF20B    | kinesin family member 20B(KIF20B) | -4.3028| 0.953258 |
| KIF2C     | kinesin family member 2C(KIF2C)   | -5.4640| 0.98209 |
| KLHL42    | kelch like family member 42(KLHL42) | -1.6637| 0.836761 |
| KNL1      | kinetochore scaffold 1(KNL1)      | -7.5343| 0.992859 |
| KNSTRN    | kinetochore localized astrin/SPAG5 binding protein(KNSTRN) | -3.7230| 0.939731 |
| KNTC1     | kinetochore associated 1(KNTC1)   | -4.5576| 0.957165 |
| LIG1      | DNA ligase 1(LIG1)                | -4.2166| 0.938967 |
| LIG3      | DNA ligase 3(LIG3)                | -1.7388| 0.839806 |
| LRRCC1    | leucine rich repeat and coiled-coil centrosomal protein 1(LRRCC1) | -2.2088| 0.871791 |
| MAD1L1    | MAD1 mitotic arrest deficient like 1(MAD1L1) | -1.6437| 0.812869 |
| MAD2L1    | MAD2 mitotic arrest deficient-like 1 (yeast)(MAD2L1) | -3.9108| 0.944761 |
| MAD2L2    | MAD2 mitotic arrest deficient-like 2 (yeast)(MAD2L2) | -2.0773| 0.863555 |
| MAPRE1    | microtubule associated protein RP/EB family member 1(MAPRE1) | -1.4912| 0.827744 |
| MARK4     | microtubule affinity regulating kinase 4(MARK4) | -1.7608| 0.840462 |
| Gene   | Description                                                                 | Z-score | P-value |
|--------|------------------------------------------------------------------------------|---------|---------|
| MASTL  | microtubule associated serine/threonine kinase like (MASTL)                  | -2.804124912 | 0.898531 |
| MCM5   | minichromosome maintenance complex component 5 (MCM5)                        | -3.171400594 | 0.922243 |
| MIS18A | MIS18 kinetochore protein A (MIS18A)                                         | -1.969925103 | 0.850172 |
| MIS18BP1| MIS18 binding protein 1 (MIS18BP1)                                          | -2.865197495 | 0.907171 |
| NCAPD2 | non-SMC condensin I complex subunit D2 (NCAPD2)                              | -2.93028383  | 0.913081 |
| NCAPD3 | non-SMC condensin II complex subunit D3 (NCAPD3)                             | -3.515551323 | 0.931072 |
| NCAPG  | non-SMC condensin I complex subunit G (NCAPG)                                | -10.44324558 | 0.994341 |
| NCAPG2 | non-SMC condensin II complex subunit G2 (NCAPG2)                             | -2.327932425 | 0.881761 |
| NCAPH  | non-SMC condensin I complex subunit H (NCAPH)                                | -8.042199962 | 0.97918  |
| NDC80  | NDC80, kinetochore complex component (NDC80)                                 | -7.372603792 | 0.996147 |
| NDE1   | nudE neurodevelopment protein 1 (NDE1)                                       | -2.201311183 | 0.867255 |
| NEDD1  | neural precursor cell expressed, developmentally down-regulated (NEDD1)      | -3.237932425 | 0.881761 |
| NEK1   | NIMA related kinase 1 (NEK1)                                                 | -1.618906452 | 0.831354 |
| NEK2   | NIMA related kinase 2 (NEK2)                                                 | -8.016334882 | 0.994521 |
| NSMCE2 | NSE2/MMS21 homolog, SMC5-SMC6 complex SUMO ligase (NSMCE2)                   | -1.967107668 | 0.834076 |
| NUF2   | NUF2, NDC80 kinetochore complex component (NUF2)                              | -10.18858432 | 0.993021 |
| NUP37  | nucleoporin 37 (NUP37)                                                       | -1.83794119 | 0.844055 |
| NUP43  | nucleoporin 43 (NUP43)                                                       | -1.788424982 | 0.852839 |
| OIP5   | Opa interacting protein 5 (OIP5)                                             | -9.044284717 | 0.982162 |
| PAPD7  | poly(A) RNA polymerase D7, non-canonical (PAPD7)                             | -1.42335585 | 0.80969 |
| PARD6B | par-6 family cell polarity regulator beta (PARD6B)                           | -3.14137613 | 0.855399 |
| PARD6G | par-6 family cell polarity regulator gamma (PARD6G)                          | -2.430080974 | 0.864597 |
| PDS5A  | PDS5 cohesin associated factor A (PDS5A)                                     | -1.70598813 | 0.846013 |
| PDS5B  | PDS5 cohesin associated factor B (PDS5B)                                     | -2.315283311 | 0.880755 |
| PHF13  | PHD finger protein 13 (PHF13)                                                | -1.482405955 | 0.814127 |
| PKN2   | protein kinase N2 (PKN2)                                                     | -1.895508529 | 0.858776 |
| POGZ   | pogo transposable element with ZNF domain (POGZ)                             | -1.381156401 | 0.815079 |
| PPP1cb | protein phosphatase 1 catalytic subunit beta (PPP1CB)                        | -1.704681815 | 0.850836 |
| PRPF40A| pre-mRNA processing factor 40 homolog A (PRPF40A)                           | -2.002939844 | 0.86174 |
| PSRC1  | proline and serine rich coiled-coil 1 (PSRC1)                                | -2.653847184 | 0.878249 |
| PTTG1  | pituitary tumor-transforming 1 (PTTG1)                                       | -8.520184893 | 0.997539 |
| ID   | Gene   | Description                                      | Log2 Fold Change | p-Value   |
|------|--------|--------------------------------------------------|-----------------|-----------|
| 5885 | RAD21  | RAD21 cohesin complex component (RAD21)          | -1.806249371    | 0.857779  |
| 5901 | RAN    | RAN, member RAS oncogene family (RAN)            | -1.220553018    | 0.802136  |
| 5925 | RB1    | RB transcriptional corepressor 1 (RB1)          | -1.836512548    | 0.855282  |
| 5932 | RBBP8  | RB binding protein 8, endonuclease (RBBP8)      | -3.432726902    | 0.927138  |
| 1104 | RCC1   | regulator of chromosome condensation 1 (RCC1)   | -2.991824603    | 0.908303  |
| 55920| RCC2   | regulator of chromosome condensation 2 (RCC2)   | -2.108664362    | 0.869573  |
| 221035| REEP3  | receptor accessory protein 3 (REEP3)            | -1.777980047    | 0.848977  |
| 80346| REEP4  | receptor accessory protein 4 (REEP4)            | -2.275161898    | 0.854941  |
| 8607 | RUVBL1 | RuvB like AAA ATPase 1 (RUVBL1)                 | -1.5113951      | 0.823037  |
| 29901| SAC3D1 | SAC3 domain containing 1 (SAC3D1)               | -2.347165267    | 0.82689   |
| 151648| SGO1  | shugoshin 1 (SGO1)                              | -6.057572753    | 0.950779  |
| 151246| SGO2  | shugoshin 2 (SGO2)                              | -4.662805762    | 0.965204  |
| 220134| SKA1  | spindle and kinetochore associated complex subunit 1 (SKA1) | -5.031273964 | 0.957875  |
| 348235| SKA2  | spindle and kinetochore associated complex subunit 2 (SKA2) | -2.482107442 | 0.893653  |
| 221150| SKA3  | spindle and kinetochore associated complex subunit 3 (SKA3) | -9.402507776 | 0.986626  |
| 7465 | WEE1   | WEE1 G2 checkpoint kinase (WEE1)                | -2.132777022    | 0.877405  |
### Table S3: Summary of ChIP-seq results of reads

| Sample         | Total reads | %GC  | Q20  | Uniq mapped rate(%) |
|----------------|-------------|------|------|----------------------|
| Fetal-H3K36me3 | 21777317    | 40.43| 90.27| 64.67                |
| Fetal-H3K4me3  | 11966862    | 40.38| 0.00 | 72.15                |
| Adult-H3K36me3| 33072780    | 49.35| 96.81| 74.68                |
| Adult-H3K4me3 | 30724003    | 54.81| 97.54| 81.41                |
Table S4: mRNA sequences of primers for PCR

| Transcripts     | Forward primer (5'->3')       | Reverse primer (5'->3')          |
|-----------------|-------------------------------|----------------------------------|
| PTTG2 (human)   | AGGAGCTACGGAGGCAAATG          | GGCAGCTGTGCAGTCACCTGTA           |
| E2F1 (human)    | TTGACCCCTCTGGATTTCTG          | CCGTGGCTGTGTGGCTGTA              |
| CDK6 (human)    | CCTCCTTCTGAATGCTTGACG         | AAGTACGGGTGATTACAGGCC            |
| CKAP2 (human)   | AACCCCAGACTCAAAGCCG           | CTACGTTTTCTGAATTACGTTCTT         |
| CKS2 (human)    | TTTTTTCCTTCGCACGCGCCAGC       | GGGTAAACATGACATGCGGT             |
| PCDH7 (human)   | ATCTGTTAACGCGTTGGGCCTG        | GAAAACGGCGTTGGCTGACT             |
| SPC25 (human)   | GAGACGCGCGTTGTTCTGGA          | ACTATGCGCCGTGCTTAACCTG           |
| H2AFX (human)   | TGGCTATGTGGACAGCAAGA          | CTTGGAATGGCCAGTTGAGT             |
| MYH6            | GACTTCCGGCGAGAGGTATCG         | GCTTGAGCCTGGATTCTGGT             |
| GPX3            | GCTTCCGGTTCCAAATGAGC          | GAGGAGGCTGGTGGCATAG              |
| TAGLN           | ATCCTATGGCATGAGCCGTG          | CAGGCTGTTCACAAACTTG              |
| ATP1A2          | GGCGGCTTCTTCACCTACTT          | TGTCCTCAGGTGACGTTGTA             |
| C1S             | CGAGGAACGTTTACTGGCT           | CCCACTACAGTTGACCCAC              |
| GAPDH (human)   | ACCACAGTCCATGCCATCAC          | TCCACACCTGTTGGCTGTA              |
| Transcripts                  | Forward primer (5'-3') | Reverse primer (5'-3') |
|-----------------------------|------------------------|------------------------|
| LncRNA ECRAR (human)        | TCTGGGTGGCTTTGGACAAG   | ACTCTCAGGCATTGCTCAACTT |
| TSPAN9-AS1 (human)          | GATCTCCTTCATCCCTCT     | GTGCTTTTAGTGTGCTT      |
| H19 (human)                 | GAAAAGCTGGAAGATGGAG    | TAAACGGGATGAATGTGCTTGC |
| NONHSAG001492 (human)       | CAACAAAGACCAAGACACATC  | ACGACAATGTGCCCTTCATCTG |
| NONHSAG039839 (human)       | GTGCTTGAATTCAGCCACAT   | GGCTGATCAGATGATCTTTC   |
| NONHSAG025186 (human)       | CTTCTCTTCCGACTCAGTT    | GCTAGCTTTTGGGAGGAT     |
| NONHSAG038579 (human)       | TGACCATGGACTTTGTGGATG  | ACACCTGGAGTGTGAGGAGT   |
| NONHSAG035519 (human)       | GTAGATCAATGCTACCAGTT   | CTCTTCAACAGTACGATACGC  |
| NONHSAG007671 (human)       | TCAGGGACGTGCATAATCAAG  | TATGCACAATGTGGCAGCAAG  |
| NONHSAG050675 (human)       | GACCACAACCTTTACCTCCAG  | GAAGCCGAATAGAGGTATCGT  |
| NONHSAG015906 (human)       | CGGCTGTGAAAGTGTTCCTCT  | TCAGCTTTCTGTGGCATCTGG  |
| NONHSAG011422 (human)       | GCCTTAGTCCAGCTGTTTCT   | ACTTGCTGAGTGGTGTGTC    |
| NONHSAG032860 (human)       | GCTAGGCATGTATTATTGTC   | GTGAACATGTGCACATGTGAG  |
**Table S6: Rat gene sequences of primers for PCR**

| Transcripts      | Forward primer (5’->3’)       | Reverse primer (5’->3’)       |
|------------------|-------------------------------|-------------------------------|
| LncECRAR (rat)   | TCAAAGCCTTAGATGGG             | CTCTGTGACAGTCCCCC            |
| Aurka (rat)      | GACGCCACCAGAGTTTAT            | CATCCGACCTTTCAATCAT          |
| Ccna2(rat)       | AGCCAGACATCACAAGCAGA         | GAATAGTGATCGTCCGTAA          |
| Ccne2(rat)       | GAGAATGTCAGAGACGCAGTA        | TTTTGGTCCAGGGTCAG            |
| Cdk2(rat)        | AAAAGGTTGGAGAAGATTG           | CGATGGTAGGGATTGAG            |
| Cdkn1a(rat)      | GAGCAAAAGATATGCCCCGTG        | CTGGCTGGCCCCTGGTTT           |
| Cdx2(rat)        | GGTGAGGTCTGCTCAGGGTG         | GGTTGATGAGGGGTTGGA           |
| E2f8(rat)        | GGGAAAGCGGGCTACTACG          | GACGGGGCATTTACAGAG           |
| Cdkn2b(rat)      | TGTGCGCTGAGGTCGGTCC          | ACGGGTTGAGGGGTTG            |
| GALN(rat)        | GTTCCCACCACACTGCCCTCA        | TCCCTTCCACTCCACTCCA          |
| Bcl2a1(rat)      | TAATGGGAGAAGATTGT             | CAGTAGTGTGGCTGGAGGAG         |
| E2F1 (rat)       | CTGCAACAACCTGCAAGGAGAG       | CCATTTGTCTGCAAGGCTT          |
| GAPDH (rat)      | GGGAAACTGTCGGCCTGAT          | GAGTGGGTGTCGCTGTGTA          |
| β-actin (rat)    | TGTCACCAACTGGGACGATA         | GGGGTGTTGAGGTCTCAAA          |
| U6 (rat)         | CTCGCTTCGGCAGCACA            | AACGCTTCACGAATTGCGT          |
Supplemental Methods

RNA-Seq Data and Mapping
The RNA-seq datasets were acquired from the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena). The RNA-seq data of fetal hearts downloaded with accession numbers SRR643778 and SRR643779, and the RNA-seq data of adult hearts downloaded with accession numbers ERR315356_1 and ERR315430_1. Clean reads were mapped to hg19 (http://genome.ucsc.edu) using SOAPaligner/SOAP2. The resulting alignments were further reconstructed using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/). The RefSeq and Ensembl transcript databases were used as the annotation references for mRNA analyses, while NONCODE v4.0 (http://www.noncode.org/NONCODERv4) was used for lncRNA annotation.

Expression Analysis of mRNAs and IncRNAs
To measure gene expression, we used the reads per kilobase transcriptome per million reads (RPKM) and an RPKM >1 was required for further analysis. To identify differentially expressed genes, we used the R-package previously implemented by the authors of NOISeq. NOISeq identifies differentially expressed genes from RNA-seq count data using a non-parametric approach that takes into account the number of reads. A probability threshold of 0.8 was used for our analysis.

Prediction of Novel Transcripts
Following alignment of candidate transcripts to a reference genome, non-overlapping transcripts were assessed for protein-coding-potential using PhyloCSF (https://github.com/mlin/PhyloCSF). We used a threshold to distinguish coding from non-coding RNAs in which transcripts with a PhyloCSF >20 were classified as mRNAs and those with a score <20 were identified as IncRNAs. The coding capacity of transcripts was predicted by CNCI (coding noncoding index, 2.0, https://github.com/www-bioinfo-org/CNCI) (Sun et al., 2013). If the value of CNCI< 0, the transcripts were considered to be noncoding.

Analysis of IncRNA Expression Patterns
We evaluated protein-coding potential of transcripts by GeneID v1.4.4
(http://genome.crg.es/software/geneid/) using transcript sequences in FASTA format under options -s and -G with eight parameters adapted for vertebrates as provided by the authors in the file GeneID.human.070123.param. PhastCons scores by chromosome were downloaded from the University of California, Santa Cruz (UCSC) genome browser (https://genome.ucsc.edu/). For each gene, scores per base for exons, introns, and promoters were summed and divided by the fragment length, which was used as the score per fragment. Next, we performed the same calculation on 50 000 randomly generated intergenic regions (size=3400 bp ± 20%). Log\(_{10}\) of the scores was plotted by category using the R package lattice and the scores of the intergenic regions were added to the three plots (exon, intron, and promoter) as a comparison.

**Hierarchical Clustering Analyses**

We used Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and JavaTreeview (http://jtreeview.sourceforge.net/) to perform cluster analysis of gene expression patterns. Differentially expressed genes were clustered by hierarchical complete linkage clustering using Euclidean distance. Prior to hierarchical clustering analyses, we performed the following steps: (1) genes covered by a single read were filtered out for lack of reliability; and (2) we configured the dataset for cluster analysis using Excel 2007(Microsoft Corporation, Redmond, WA, USA). Specifically, all data values (x) were multiplied by log\(_{2}\)(x) to log transform the data. Next, we subtracted the column-wise mean from the values in each data column so that the mean value of each column was zero (0).

**Functional Analysis**

Our gene ID lists were submitted online to the DAVID Bioinformatics Resource (https://david.ncifcrf.gov/) for Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. A hypergeometric test with Benjamini and Hochberg false discovery rate (FDR) was performed using default parameters to adjust the \(P\) value for multiple comparisons. Enrichment was defined as significant for \(P\) values <0.05. Prediction of functional RNA motifs was performed using RegRNA2.0 (http://regrna2.mbc.nctu.edu.tw/).
Visualization using Integrative Genomics Viewer

Case studies were explored using Integrative Genomics Viewer (IGV) version 2.3.32 (\texttt{www.broadinstitute.org/igvhttps://www.broadinstitute.org/igv/}). To visualize the read coverage signal map of fetal and adult hearts in IGV, BAM files were generated in a compatible IGV format.

Chromatin-Immunoprecipitation Sequencing

Chromatin-immunoprecipitation (ChIP)-sequencing (ChIP-seq) data of fetal and adult human cardiac tissues were obtained from ENA (\texttt{http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc}) under accession numbers GSM878630 and ERP003613. H3K4me3 and H3K36me3 were used as markers (Online Table S2). A marker was considered present if a non-empty intersection was detected between the transcription start site (TSS) region and a marker peak in any replicate. Intersections were detected using the window command of BEDTools version 2.17.0 with option \texttt{-w 1000} (\texttt{http://bedtools.readthedocs.io/en/latest/}).

Correlation of Expression Between lncRNAs and Neighboring Coding Genes

The coordinates of lncRNAs were compared to reference data of coding genes. If the coordinates of the lncRNA overlapped with a known gene (at least 1 bp), this gene was considered as the closest overlapping gene. If there was no overlap with a known gene, the closest gene was selected and classified as upstream or downstream depending on its position. The correlation of expression was calculated between the lncRNA and the closest coding gene using Pearson correlation.

Cis-Gene Regulation and Trans-Gene Regulation

Numerous studies have shown that lncRNAs act through regulating transcriptional expression of its neighbor or proximal mRNA/protein coding genes. If the coordinates of the lncRNA located within 10 kb upstream and downstream of a coding gene, this gene was considered as a neighbor gene; if the coordinates of the lncRNA located either at least 1 megabase apart or present on different chromosomes, this gene was considered as proximal
genes. A true \textit{cis} effect was defined as lncRNA affect the expression of neighbor genes, and the \textit{trans} effect was of was defined as lncRNA affect the expression of proximal genes.

\textbf{Co-expression analysis between LncRNA and mRNA}

To explore the potential Co-expression between LncRNA and differentially expressed mRNA, Pearson correlation coefficients were used. The lncRNA-mRNA pairs with absolute value of correlation coefficients over 0.9 were constructed regulatory network by using Cytoscape 3.0. Those mRNA ID lists were submitted online to the DAVID Bioinformatics Resource (https://david.ncifcrf.gov/) for Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses.

\textbf{Overexpression of ECRAR}

LncRNA ECRAR sequence was cloned into adenovirus expression vector GV315. Adenoviruses were generated using adenoviral expression systems performed by GENECHEM Inc. (Shanghai, China). Transfection efficiency [multiplicity of infection (MOI)=10] was determined by GFP expression to be over 90% and overexpression efficiency was measured by qPCR up to 4-fold (Figure S10A-B).

\textbf{ECRAR Knockdown}

For ECRAR knockdown experiments, the following target sequences were used: (1) shRNA-1: 5′- GGTCTGGACCTTCAATCAA -3′; (2) sRNA-2: 5′-GGGAGATCTCAAGTTTCAA -3′; (3) sRNA-3: 5′- GGTAAGATGTATTTCATGAA -3′; Each of the 3 shRNA was cloned into pDC316-siRNA-EGFP Vector. Adenoviruses were generated using adenoviral expression systems performed by GENECHEM Inc. (Shanghai, China). Rat ventricular CMs were transfected with sh-ECRAR adenovirus or sh-null adenovirus for 48 h. Transfection efficiency (MOI=10) was determined by GFP expression to be over 80%. RT-qPCR confirmed that ECRAR was downregulated in the sh-ECRAR group (Figure S17A).
**Isolation and Culture of Cardiomyocytes**

Cardiomyocytes (CMs) from 1-day-old (postnatal day 1; P1) or 7-day-old (P7) Wistar rats (purchased from the Experimental Animal Center of Southern Medical University) were isolated as previously described. Briefly, rats were anesthetized by 2% isoflurane inhalation, ventricles were separated from their atria, cut into pieces, and digested in 0.25% trypsin at 4°C for 12-16h. Digestion was repeated 2–3 times at 37°C for 15 min, and the supernatant was collected and mixed with fetal bovine serum (FBS) (Gibco, Life Technologies, USA). The supernatant was collected and centrifuged, and cells were re-suspended in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma, USA). The cell suspension was passed through a 40-mm cell strainer (BD Falcon) and then seeded onto uncoated 100-mm plastic dishes for 90 min at 37°C and 5% CO2 humidified atmosphere. Then, the cell suspension was seeded onto uncoated 100-mm plastic dishes for 90 min at 37°C and 5% CO2 humidified atmosphere again. The supernatant, which is mostly composed of CMs, was then collected and pelleted by centrifugation. Next, cells were suspended in antibiotic-free media, counted, and plated at the appropriate density (supplementary Figure S10A-B). The purity of ventricular CMs prepared by this procedure was >90%. CMs were cultured via differential adhesion. All cells were incubated in DMEM with 10% FBS in a thermostat-controlled incubator at 37°C and 5% CO2.

**Isolation and Culture of Neonatal Cardiac Fibroblasts**

Cardiac fibroblasts (CFs) were isolated from rats as previously described. Excised hearts were rinsed in cold Hank’s balanced salt solution (HBSS), minced, and digested with type II collagenase and pancreatin at 37°C for 15 minutes. The first digestion was discarded. A second digestion was performed and the collagenase medium containing CFs was collected, centrifuged, and resuspended in DMEM with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Digestion was repeated 5–6 times until the digestion fluid became clear. Cells were plated in 60-mm dishes and allowed to attach for 60 min, and the media
was then changed to remove CMs and endothelial cells. Isolated CFs were washed twice with phosphate-buffered saline (PBS) and cultured for transfection experiments.

**Volume Analysis of Isolated Cardiomyocytes**

As previously described, the isolated cardiomyocytes were stained with cTnT and DAPI. 3D images of cardiomyocytes were obtained using a Zeiss confocal LSM700 microscope (633Plan-Apooilobjec-tive), and the individual cardiomyocyte volume was measured by using the Imaris 8 (Bitplane) 3D image processing software program. The volumes were estimated with the following equation: \( V (\mu m^3) = L(\mu m) \times W(\mu m) \times H(\mu m) \).

**Estimation of Total Number of Cardiomyocytes**

As previously described, tissue pieces (1–2mm diameter) from the left Ventricle were sampled. CM nuclei were stained with antibodies against PCM-1, and nuclei were stained with DAPI. The cytoplasm of CM was stained with cTnT. To facilitate the identification of the cell borders, Wheat germ glutinin (WGA) was added. Using the CAST software, the serial sections could be analyzed all together. In each block, the number of CM s was calculated, and Serial sections were counted to estimate the total number of CM s. For each animal, 4 different tissue blocks were analyzed,

**Microarrays**

**Samples**

Following CM transfection with Ad-ECRAR or Ad-null for 72 h at MOI=10, total RNA of four samples was isolated with TRIzol. RNA quantity and quality were measured using a NanoDrop ND-1000. RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

**Microarrays**

Rat Gene Expression Arrays (4×44K), which provide full coverage of rat genes and transcripts, were used from Agilent and microarray experiments were performed by KangChen Bio-tech.
RNA labeling and array hybridization

Sample labeling and array hybridization were performed according to the One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies). Briefly, total RNA from each sample was linearly amplified and labeled with Cy3-UTP. The labeled cRNAs were purified using RNeasy Mini Kit (Qiagen) and the concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using a NanoDrop ND-1000. One microgram of each labeled cRNA was fragmented by adding 11 μL 10× Blocking Agent and 2.2 μL of 25× Fragmentation Buffer. The mixture was heated to 60°C for 30 min followed by the addition of 55 μL 2× GE Hybridization buffer to dilute the labeled cRNA. One-hundred microliters of hybridization solution was dispensed onto the gasket slide, which was then sandwiched with the gene expression microarray slide. The slides were incubated for 17 h at 65°C in a Hybridization Oven (Agilent). Hybridized arrays were washed, fixed, and scanned using a G2505C DNA Microarray Scanner (Agilent).

Analysis

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). After quantile normalization of the raw data, genes that have at least 1 out of 4 samples with flags in Detected (“All Targets Value”) were chosen for further data analysis. Differentially expressed genes with statistical significance between the two groups were identified through Volcano Plot filtering. Differentially expressed genes between the two samples were identified through Fold Change filtering. Hierarchical Clustering was performed using R scripts. GO analysis and KEGG pathway analysis were performed in the standard enrichment computation method.

5' and 3' Rapid Amplification of cDNA Ends (RACE)

We used the 5'-RACE and 3'-RACE analyses to determine the transcriptional initiation and termination sites of lncRNA-ECRAR using a Invitrogen™ 3' RACE System for Rapid Amplification of cDNA Ends (Catalog no.18373-019, ) and Invitrogen™ 5' RACE System
for Rapid Amplification of cDNA Ends (Catalog no.18374-058) according to the
manufacturer’s instructions. The gene-specific primers of lncRNA-ECRAR used for the
PCR of the 5'RACE analysis were 5'- TGGTCTTTACAGACTTTTCTGTAGCTCTG -3'
(5'RACE) and primers used for 3'RACE analysis were 5'- CAGAGCTACAGAAAAGTCTG
-3'.

Cell Proliferation Assay
The cell proliferation assay was performed as described previously.7, 8 For in vitro
experiments, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with
0.5% Triton X-100 in PBS for 10 min, followed by 30 min blocking in 1% bovine serum
albumin (BSA) (Sigma). Cells were then incubated overnight at 4°C with the following
primary antibodies diluted in blocking solution: a mouse monoclonal antibody against
sarcomeric α-actinin (1:200, ab9465, Abcam, Cambridge, UK) or a rabbit antibody against
Ki-67 (1:200, 9129, CST, USA), pH3 (1:100, ab154206, Abcam), aurora B (1:600, ab2254,
Abcam), ANP (1:400, ab40791, Abcam), or α-SMA(1:1000, ab5694, Abcam) followed by a
PBS wash and incubated for 2 h with the respective secondary antibodies conjugated to Alexa
Fluor-488, Alexa Fluor-555, or Alexa Fluor-647 (1:200, Abcam). The Click-iT® EdU
Imaging Kits (Life Technologies, USA) to detect 5-ethynyl-2′-deoxyuridine (EdU)
incorporation were used according to the manufacturer’s instructions. Finally, cells were
stained with Hoechst 33342 (Life Technologies).

For in vivo experiments, sections of rat hearts were deparaffinized, rehydrated, and then
underwent antigen retrieval by boiling in a sodium citrate solution for 20 min. Slides were
processed for immunofluorescence as described above for cultured cells. After adenoviral
vector transfection for 2 days, EdU was administered intraperitoneally in rats
every 2 days for a total period of 10 days at which time hearts were collected.

Cells were identified as proliferating if positive for the proliferation marker of cell cycle
re-entry (Ki-67), DNA synthesis (EdU), mitosis (pH3), and cytokinesis (Aurora B) as
described previously.9-12 CMs were distinguished from other cells (e.g., fibroblasts) by
positivity for sarcomeric α-actinin or cTnT. The fibroblasts were label with vimentin CD90
and Sca-1. The number of positive cells reported was calculated as a sum of the number of
positive cells from five randomly chosen fields in each animal using a FluoView confocal microscope (Olympus Corporation, Tokyo, Japan). Inhibits phosphorylation of ERK1/2 were performed by using PD0325901 (2 µM, Tocris Bioscience) was added in the presence of Ad-ECRAR or Ad-null.

Evaluation of infarct size and fibrosis
The infarct size and fibrosis area were evaluated as described previously. At 14 days and 60 days post-MI, adult rat hearts were fixed in 10% formalin, dehydrated, and embedded in paraffin. Then, 4–6-um sections were cut for histological studies. To evaluate infarct size, the cut heart sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC) where white staining indicates the infarcted zone. To evaluate the extent of cardiac fibrosis, the heart sections were stained with Masson’s trichrome stain in which blue staining indicates collagen and red staining indicates myocardium. The percent area of cardiac fibrosis was determined from five random images obtained from each animal and calculated as the ratio of the Masson’s trichrome-stained area to the total optical field using Image J (https://imagej.nih.gov/ij/).

Echocardiography
Cardiac function was evaluated blindly using echocardiography before MI, and at 1 day, 14 days, 30 days, and 60 days after MI induction as described previously. M-mode echocardiography was performed to measure the LV fractional shortening (LVFS), LV end-diastolic diameter (LVEDd), and LV end-systolic diameter (LVESd). Fractional shortening (FS) was calculated as (LVEDd-LVESd)/LVEDd×100%; left ventricular end diastolic volume (LVEDv) was calculated as 7.0×LVEDd³/(2.4+LVEDd); left ventricular end systolic volume (LVESv) was calculated as 7.0×LVESd³/(2.4+LVESd); and left ventricular ejection fraction (LVEF) was calculated as (LVEDv-LVESv)/LVEDv×100%.

Assessment of the capillary density
As previously described, the capillary and arteriole densities in the peri-infarct and infarct zones were quantified using immunohistochemical and immunostaining analysis, by staining with anti-rat CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or α-SMA. The
capillary densities are calculated as the number of capillaries per unit area image from 5 randomly chosen x 200 fields in each animal using Olympus BX51 microscope (Olympus Corporation). The capillary and arteriole density was calculated according to the percentages of positively stained areas in relation to those of the whole view field.

**RNA Extraction and qRT-PCR Analysis**

Total RNA was extracted from CMs using the E.Z.N.A.R Total RNA Kit II (Omega Biotek, Norcross, GA, USA), and cDNA was synthesized using a PrimeScript™ RT Master Mix (TaKaRa, Dalian, China). PCR analysis was performed using SYBRs Premix Ex Taq™ Kit (TaKaRa), and amplified PCR products were quantified and normalized using GAPDH or U6 as a control. PCR cycle parameters for ECRAR expression were as follows: an initial denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, and melting curve generation. The primer pairs used in this study are provided in Online Table S3 to S5.

**Western Blot Analysis**

Total protein was obtained from cultured CMs or heart ventricle tissue using a lysate containing RIPA lysis buffer and protease inhibitor. Protein samples (100 μg) were electrophoresed using 10% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking membranes with 5% BSA for 1 h at room temperature, they were incubated with the primary antibody in 5% BSA overnight at 4°C. The primary antibodies were against pH3 (1:1000, ab154206, Abcam), p-ERK1/2 (1:1000, #4370, cell signaling technology), t-ERK1/2 (1:1000, ab109282, abcam), cyclin D1 (1:1000, ab40754, abcam), cyclin E1 (1:1000, ab71535, Abcam) and E2F1 (1:1000, ab179445, Abcam), whereas antibodies against β-actin (1:5000 dilution; ab6276; Abcam) and GAPDH (1:200 dilution, sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as loading controls. Two secondary antibodies were used. One was labeled with horseradish peroxidase (HRP) at a dilution of 1:3000 and the other Alexa Fluor®680 (Abcam) was used at a dilution of 1:10 000 dilution; both were incubated for 1 h at room temperature. Quantitation of protein expression levels was analyzed using Odyssey version 1.2 (LI-COR), and the expression of
each protein was normalized against β-actin or GAPDH expression. Data were quantified by
densitometry using Image J (https://imagej.nih.gov/ij/). For CCND1 inhibition, the following
target sequences were used: GCUAUUGGAGGAUCAGUUUTT; For CCNE1 inhibition, the
following target sequence were used: GCCAUGUUGUCUGAACAAATT.

RNA FISH and co-localization analysis

First, CMs were cultured on a glass coverslip, and then fixed in 4% paraformaldehyde for 30
min. Next, cells were permeabilized with 0.1% Triton X-100 for 10 min followed by three
washes with PBS and treated with pre-hybridization buffer (2× saline-sodium citrate)
containing 10% formamide. Hybridization was performed using a similar buffer, but with the
addition of competitor RNA (tRNA) and competitor protein (BSA) to reduce background.
The FITC-labeled ECRAR probe was synthesized by Bersin Biotechnology Company
(Guangzhou, China), which was diluted in 200 μL hybridization buffer, was deposited on a
surface in a humid dark chamber. The glass coverslip was then placed face down on the drop
and incubated at 37°C for 12 h. Following incubation with the ECRAR probe, cells were
washed five times with PBS and stained with Hoechst (Life Technologies) for 2 h at 37°C.
After the cells were washed several times, the coverslip was mounted onto a microscope slide
using an anti-fade mounting medium. For co-localization analysis, after RNA-FISH, cells
were again fixed for 5 min in 2% formaldehyde, then subjected to immunofluorescence.

Isolation of Cell Nuclear/Cytoplasmic Fractions

Subcellular cytoplasmic and nuclear RNA fractions were extracted using NE-PER Nuclear
and Cytoplasmic Extraction Reagents (Thermo Fisher, Grand Island, NY, USA) according to
manufacturer’s instructions. Cells were washed with ice-cold PBS twice then lysed in
ice-cold CER (Cytoplasmic Extraction Reagent), and after a maximum speed centrifugation
(~16 000×g), the resulting supernatant was collected as the cytoplasmic fraction. Next, the
insoluble fraction was lysed in ice-cold NER (Nuclear Extraction Reagent) for 40 min, and
after a maximum speed centrifugation (~16 000×g), the supernatant was collected as the
nuclear fraction. We performed qRT-PCR using the SYBR Premix Ex Taq™ Kit (TaKaRa) to amplify localized ECRAR. The qRT-PCR conditions were as follows: an initial denaturation of 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, with a final extension at 72°C for 10 min.

RNA Pull-Down Analysis

The RNA pull-down assay was performed as described previously. Biotinylated ECRAR and antisense ECRAR were mixed with proteins obtained from P7 CMs overnight at 4°C. Biotinylated ECRAR-protein complexes were purified using streptavidin-agarose for 4 h at 4°C. The proteins were then eluted from the complex and detected by western blot analysis. Next, proteins were precipitated and diluted in 100 μL protein lysis buffer. One-shot mass spectrometry (MS) analyses were then performed to analyze the purified nuclear proteins.

Nanospray Liquid Chromatography–Tandem MS

Different bands from RNA pull-down were excised, followed by protein elution and digestion. Digests were analyzed by nano-ultra-performance liquid chromatography–electrospray ionization tandem MS.

RNA Immunoprecipitation Analysis

Biotin-labeled p-ERK1/2 (1:1000, #4370, cell signaling technology) and IgG antibodies were added to cell extracts and incubated overnight at 4°C. Streptavidin-coated magnetic beads were then added and incubated for 4 h at 4°C. Magnetic beads were pelleted, washed, and re-suspended in 1 mL TRIzol. Isolated RNA was reverse transcribed to cDNA and then analyzed by qRT-PCR. The PCR cycling parameters for ECRAR enrichment were as follows: an initial denaturation of 95°C for 5 min followed by 40 cycles of 94°C for 10 s and 65°C for 40 s, with a final extension at 72°C for 10 min.

Luciferase Reporter Assay

ECRAR wild type (pGL3-ECRAR-wt) and a mutant derivative devoid of the E2F1 binding site (pGL3-ECRAR-mut) were cloned downstream from the coding region of the luciferase gene. Target sequences were 5’-TCTGGCGCCAGC-3’. For luciferase reporter assays,
AC16 human cardiomyocytes (HZ-CC337712, American Type Culture Collection, USA) were added to the overexpression vector of E2F1, and then transfected with the indicated luciferase constructs as described above. Transfection was performed using Lipofectamine 2000 (ThermoFisher Scientific, USA) according to the manufacturer’s instructions. Firefly and Renilla luciferase activities were measured using a Dual Luciferase Reporter Assay System (Promega, USA) and each well had three replicates.

**Chromatin Immunoprecipitation with Quantitative PCR (ChIP-qPCR)**

The ChIP assay was performed using Pierce™ Agarose ChIP Kit (#26156; ThermoFisher Scientific) according to the manufacturer’s standard protocol. The AC16 human CMs were fixed in 1% formaldehyde for 30 min at room temperature followed by DNA fragmentation by sonication at 3s/10s for 30 times. Subsequently, chromatin was immunoprecipitated with biotin-labeled E2F transcription factor 1 (E2F1) antibody. A positive control antibody (RNA polymerase II/RII), a negative control normal human IgG, and GAPDH primers were used as controls. Purified chromatin was quantified by qRT-PCR using SYBRs Premix Ex Taq™ Kit (TaKaRa using the following primer pairs: Forward: 5′-CCGCTCCGCGGTGCCAGCGC-3′; and Reverse: 5′-CCTTACATTAGATTCTGACCCTG-3′. Fold enrichment was quantified using qRT-PCR and calculated as a percentage of Input chromatin (% input).

**Time-lapse videos**

After the P7 CMs transfected with Ad-ECRAR or Ad-null for 48h, the CMs were labeled with TMRE (tetramethylrhodamine, ethylester), a fluorescent dye that labels mitochondria. Then imaged for 12-h at 10 min intervals; Live-cell imaging was performed using a Delta Vision Elite system (Applied Precision) in inverted microscope (Leica SP9), running Soft WorX6.0. Time-lapse imaging was carried out for 12h at 10 min intervals, and acquired at 10 magnification (10/0.3 NA objective) by a Cool Snap HQ2CCD (charge-coupled device) camera (Roper Scientific).
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