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Original Article

Long non-coding RNA BANCR promotes interferon-β-induced cardiomyocyte apoptosis by targeting signal transducer and activator of transcription 1 *in vitro*

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Abstract: Myocardium functions as an immune organ, and myocardial ischemia-reperfusion (I/R) is known to initiate myocardial innate immune response to induce myocardial injury. However, the mechanisms underlying interferon-β (IFN-β)-mediated myocardial injury during I/R and whether long non-coding RNAs (lncRNAs) are involved in IFN-β-mediated myocardial injury remain unknown. This study identified that I/R significantly induced IFN-β expression in induced pluripotent stem cell-derived cardiomyocytes, and IFN-β further enhanced I/R-induced myocardial apoptosis. Furthermore, it was demonstrated that the lncRNA BRAF-activated non-coding RNA (BANCR) was highly expressed in cardiomyocytes, and BANCR-knockdown suppressed signal transducer and activator of transcription 1 (STAT1) phosphorylation and IFN-β-induced cardiomyocyte apoptosis. Furthermore, it was identified that BANCR specifically interacted with STAT1 to promote IFN-β-STAT1 signaling and enhanced the expression of pro-apoptotic interferon stimulated genes. Overall, the present study reports that lncRNA BANCR promotes IFN-β-mediated cardiomyocyte apoptosis following I/R injury by interacting with STAT1, suggesting lncRNA BANCR is involved in IFN-β-induced cardiomyocyte apoptosis.

Keywords: Long non-coding RNA BRAF-activated non-coding RNA, myocardial infarction, interferon-β, signal transducer and activator of transcription 1

Introduction

Myocardial ischemia/reperfusion (I/R) is a key complication of reperfusion therapy for myocardial infarction [1]. Myocardial I/R usually triggers oxidative stress, energy dysfunction, and an inflammatory reaction, eventually leading to cardiomyocyte apoptosis [2, 3]. Until now, great advances in the investigation of pathophysiologic mechanisms of myocardial I/R injury and exploration of numerous potential therapeutic targets and cardioprotective drugs have been achieved [4, 5]. However, the pathologic mechanisms of myocardial I/R injury are not completely clear and need to be explored further.

Type I interferons (IFNs), including IFN-α and IFN-β, serve important roles in immune response against viral and bacterial infections, and cancer, whereas excessive type I IFN production can cause autoinflammatory diseases [6-8]. Excretive type I IFNs bind to cellular surface interferon receptors (IFNRs) to activate Janus kinase (JAK)-signal transducer and activator of transcription (STAT) 1/2 signaling to amplify immune responses, resulting in induction of numerous interferon-stimulated genes (ISGs) [9]. Recent studies have reported that myocardium functions as an immune organ, and I/R initiates myocardial innate immune responses through Toll-like receptors-NF-κB and interferon regulatory factor 3 (IRF-3) signaling to induce pro-inflammatory and immunomodulatory cytokine expressions [10-12]. In addition, IRF-3 and type I IFNs have been demonstrated to fuel a fatal response to myocardial infarction [11]. However, to the best of our knowledge, the mechanisms underlying type I IFN-mediated myocardial infarction during I/R are still not completely understood.

Long non-coding RNAs (lncRNAs), over 200 nucleotides, with limited or no protein coding properties, serve essential roles in various pathologic and physiologic processes, including cell apoptosis, differentiation, and metabolism [13-15]. Many studies report that lncRNAs act as master regulators in myocardial I/R injury and cardiovascular diseases [16, 17]. For example, lncRNA H19 alleviates myocardial I/R injury by targeting microRNA (miR)-22-3P [18]. lncRNA NEAT1 ameliorates myocardial injury by controlling the TLR2/NF-κB signaling pathway [19]. lncRNA-ROR aggravates myocardial I/R injury [20]. lncRNA BRAF-activated noncoding RNA (BANCR) facilitates vascular smooth muscle cell proliferation and migration during the development of numerous cardiovascular diseases [21]. However, whether lncRNAs are involved in type I IFN-mediated myocardial injury during I/R is still unclear.

The present study used human induced pluripotent stem cell-derived cardiomyocytes (iPS), which are widely used to explore the pharmacology and toxic effects of drugs on heart cells [22], and are widely used for investigating cardiac regenerative therapy [23], treated with oxygen and glucose deprivation followed by reperfusion (OGD/R) to mimic myocardial I/R injury *in vitro*. The results demonstrated that I/R significantly induced IFN-β expression in cardiomyocytes, and IFN-β further strengthened I/R-induced cardiomyocyte apoptosis. Furthermore, lncRNA BANCR was highly expressed in cardiomyocytes, and BANCR-knockdown suppressed STAT1 phosphorylation and IFN-β-induced cardiomyocyte apoptosis. Furthermore, it was identified that BANCR promoted IFN-β-STAT1 signaling and enhanced pro-apoptotic ISG expression through interacting with STAT1, suggesting lncRNA BANCR is involved in IFN-β-induced cardiomyocyte apoptosis.

Materials and methods

Cell culture and OGD/R model

iPS cell-derived cardiomyocytes were purchased from Takara Bio, Inc. (catalog no Y50015; MiraCell™ Cardiomyocytes). Cells were maintained in MiraCell CM culture medium (TAKARA, Japan) at 37°C with 5% CO_2. To stimulate I/R, cardiomyocytes were kept in a hypoxic chamber containing 5% CO_2 and 95% N_2 for 12 h at 37°C with serum- and glucose-deficient DMEM (Gibco, Carlsbad, CA, USA), and reoxygenated for the indicated times under normal conditions (5% CO_2) with DMEM containing 10% FBS (Gibco, USA).

TUNEL assay

iPS cell-derived cardiomyocytes cultured in 96-well plates were stimulated with OGD/R or stimulated with recombinant human IFN-β protein (1,000 IU/ml, R&D Systems, Inc.), or pretreated with anti-IFN α/β receptor 1 antibody (anti-IFNAR1; Abcam, USA) for 2 h and then stimulated with OGD/R or IFN-β protein for the indicated durations. Cellular apoptosis was detected using a TUNEL Apoptosis Detection kit (Vazyme Biotech Co., Ltd.), according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

iPS cell-derived cardiomyocytes cultured in 96-well plates were treated with OGD/R for the indicated durations. Secreted IFN-β expressions in cell culture supernatants were determined using a Mouse IFN-β ELISA kit (Abcam), according to the manufacturer's instructions.

Reagents, small interfering RNA (siRNA) and adeno-associated viral vector

The sequences of siRNA against BANCR (si-BANCR) were as follows: 5'-AGUUUCAACAUGAUGUUUCUG-3' and 5'-GAAACAUCAUGUUGAAACUCU-3'. Control siRNA (siNC) had the following sequences: 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'. siRNAs were synthetized by Shanghai GenePharma Co., Ltd. siRNAs or plasmids were transfected into cardiomyocytes cells for 24 h using Exfect Transfection Reagent. Recombinant adeno-associated BANCR overexpression viral vector (AAV-BANCR) or control adeno-associated viral vector (AAV-NC) were provided by Shanghai GenePharma Co., Ltd. A specific STAT1 activation inhibitor, fludarabine, was purchased from TargetMol and used at a concentration of 50 μM.

LncRNA-sequencing (LncRNA-seq)

iPS cell-derived cardiomyocytes were stimulated with recombinant human IFN-β protein (1,000 IU/ml, R&D Systems, Inc) or not for 6 h,

and then total RNA was extracted. Sequencing was performed with the Illumina HiSeq 2500 (Guangzhou RiboBio Co., Ltd.). RNA-seq data were aligned to the Ensembl v73 transcript annotations using bowtie and RSEM, as previously described [24].

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from each sample, and then reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara). qPCR was performed with AceQ qPCR SYBR Green Master mix using the 7500 real-time PCR system (Applied Biosystems) [25]. The qPCR primers were as follows: AK03516 forward, 5'-TACCCGAGACTTACCGATGCACT-3' and reverse, 5'-CCAGTCACTCTTGCCTACCAT-3'; GASL1 forward, 5'-CATGTTCCAATATGATTCCACC-3' and reverse, 5'-GATGGGATTTCCATTGATGAC-3'; MALAT1 forward, 5'-AGAGGGAAGAGCAAATGTGTCA-3' and reverse, 5'-CTGTGTTTCCTTGTCCGACCT-3'; BANCR forward, 5'-ACAGGACTCCATGGCAAACG-3' and reverse, 5'-ATGAAGAAAGCCTGGTGCAGT-3'; SAP30L-AS1 forward, 5'-ATCTCGGCTCACCGCAACCT-3' and reverse, 5'-AATGACCCAAATGCACGTCCT-3'; OIP5-AS1 forward, 5'-GGTCGTGAAACACCGTCG-3' and reverse, 5'-GTGGGGCATCCAGGGT-3'; CTB-171A8.1 forward, 5'-TGAAGTCCACCTGGAGACCT-3' and reverse, 5'-CACACGTGGATGGGAGCAGC-3'; SNHG1 forward, 5'-AGGCTGAAGTTACAGGTC-3' and reverse, 5'-TTGGCTCCCAGTGTCTTA-3'; MEG3 forward, 5'-CGCTCACAAAGGCACGTGGG-3' and reverse, 5'-CCAGGTGATGGCTTCCGCCC-3'; TUG1 forward, 5'-CTGAAGAAAGGCAACATC-3' and reverse, 5'-GTAGGCTACTACAGGATTTG-3'; CD95 forward, 5'-TCTGGTTCTTACGTCTGTTGC-3' and reverse, 5'-CTGTGCAGTCCCTAGCTTTCC-3'; TNF-α forward, 5'-GAGGCCAAGCCCTGGTATG-3' and reverse, 5'-CGGGCCGATTGATCTCAGC-3'; NOS2 forward, 5'-TCATCCGCTATGCTGGCTAC-3' and reverse, 5'-CCCGAAACCACTCGTATTTGG-3'; GAPDH forward, 5'-TCAACAGCAACTCCCACTCTTCCA-3' and reverse, 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACATATACT-3' and reverse, 5'-ACGCTTCACGAATTTGCGTGTC-3'. The obtained data were normalized to GAPDH or U6 expression levels in each sample.

Cellular fractionation and western blotting

iPS cell-derived cardiomyocytes were transfected with si-BANCR or si-NC, or infected with AAV-BANCR or AAV-NC (MOI=5) for 24 h, and then treated with recombinant human IFN-β protein (1,000 IU/ml) or not for the indicated time. The nuclear fraction of cells was extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime), and then was subjected to SDS-PAGE and immunoblotting, as previously described [25]. Primary antibodies, including rabbit-anti-STAT1 (Cell Signaling Technology), rabbit-anti-phosphorylated (p)-STAT1 (Cell Signaling Technology), rabbit-anti-STAT2 (Cell Signaling Technology), rabbit-anti-p-STAT3 (Cell Signaling Technology), rabbit-anti-GAPDH (Abcam), and rabbit anti-Lamin B1 antibody (Abcam) were used in this study.

RNA pull-down and mass spectrometry

RNA pull-down assays were performed as previously described [26]. Briefly, a biotin-labeled lncRNA-BANCR probe and antisense BANCR probe (as a control) were transcribed *in vitro* with the Biotin RNA Labeling mix (Roche Diagnostics, Inc.) using mMESSAGE mMACHINE kit (Ambion; Thermo Fisher Scientific, Inc.). Biotinylated RNAs were treated with RNase-free DNase I and then purified. Samples were incubated at room temperature for 15 min to immobilize RNA on the streptavidin dynabeads, then supernatant was removed and beads were washed with wash buffer. Nuclear lysates were added to beads with immobilized RNA, and then incubated on a turning wheel overnight at 4°C. Beads were washed five times and boiled in SDS buffer. lncRNA-BANCR interacting proteins were analyzed by SDS-PAGE and the gel was then stained with the Fast Silver Stain kit (Beyotime Institute of Biotechnology). Proteins were identified by reverse-phase liquid chromatography coupled with tandem mass spectrometry (ACQUITYTM UPLC-QTOF).

RNA-binding protein immunoprecipitation (RIP)

RIP was performed using Magna RIP kit (EMD Millipore), as previously described [25]. Briefly, cells were treated with IFN-β protein, lysed with RIP lysis buffer, then incubated with antibodies against STAT1 for 24 h. After immobilizing magnetic bead-bound complexes with a magnetic separator (EMD Millipore), the supernatant was used to extract RNAs with PCA (phenol: chloroform: isoamyl alcohol) reagent at a ratio of

125:24:1, and then reverse-transcribed into cDNA. Finally, qPCR was performed.

Statistical analysis

The data are presented as mean ± standard error of the mean, and all statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc). The differences between two groups were analyzed using unpaired Student's t-test. $P<0.05$ was considered a significant difference.

Results

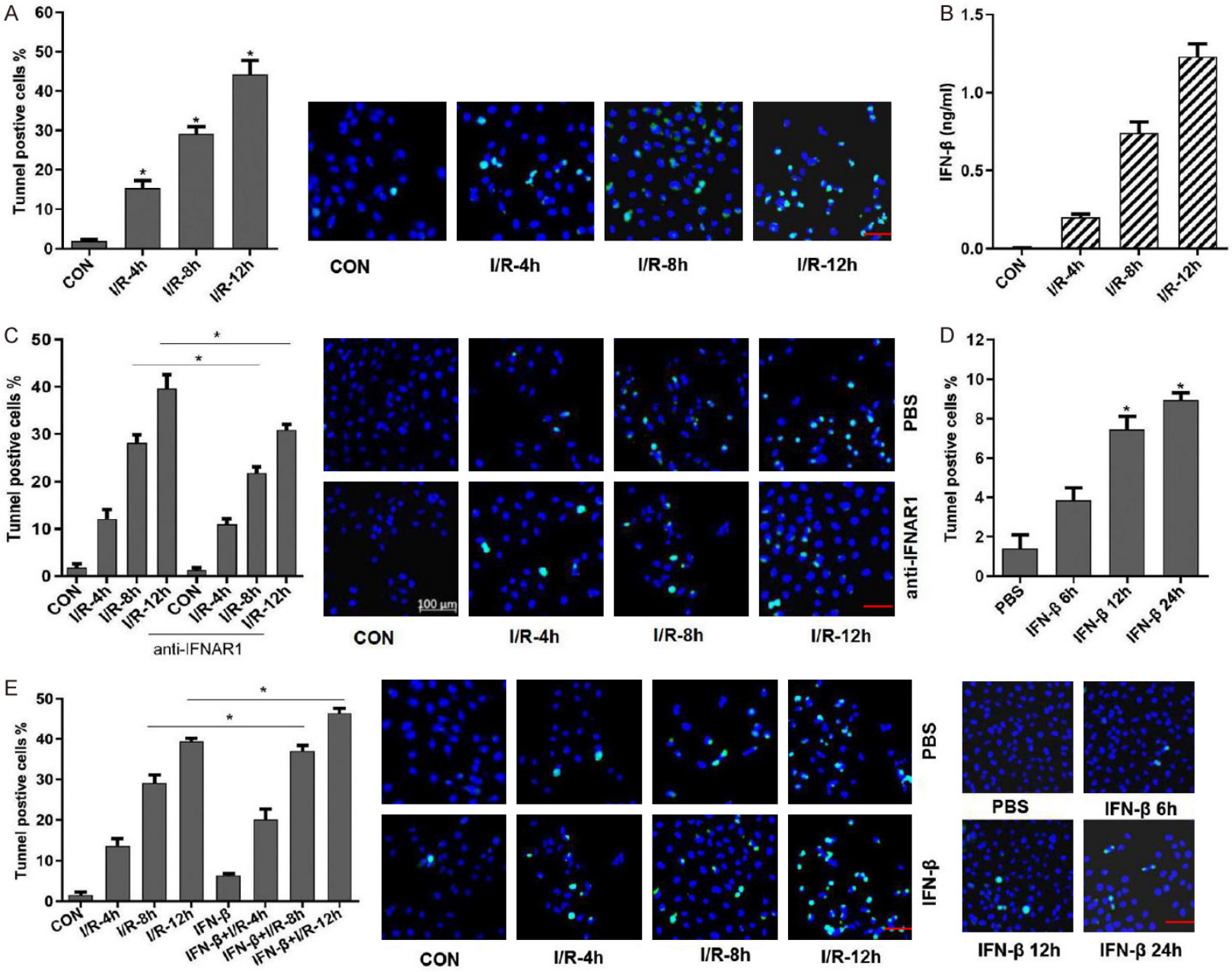
IFN-β, which is induced by I/R in cardiomyocytes, strengthens I/R-induced myocardial injury

To investigate whether IFN-β is involved in I/R-induced myocardial injury, OGD/R induced iPS cell-derived cardiomyocytes were used to construct a myocardial I/R injury model *in vitro*. As presented in **Figure 1A** and **1B**, apoptotic cardiomyocytes progressively increased during the process of I/R injury, and I/R injury significantly induced IFN-β expression in cardiomyocytes, which was consistent with previous studies, in which the myocardium was shown to act as an immune organ to trigger innate immune response [10, 11]. Furthermore, the present study explored whether IFN-β induced by cardiomyocyte I/R is related to I/R-triggered myocardial apoptosis. The results demonstrated that pretreatment with anti-IFNAR1 antibody, which prevents excretive type I IFNs binding to cellular surface IFNRs of cardiomyocytes to activate JAK-STAT1/2 signaling, significantly suppressed I/R-triggered myocardial apoptosis (**Figure 1C**). In addition, IFN-β treatment could not only induce myocardial apoptosis (**Figure 1D**), but also further strengthened I/R-induced myocardial apoptosis (**Figure 1E**). Consistently, pretreatment with anti-IFNAR1 antibody could also significantly suppress IFN-β-triggered myocardial apoptosis (**Figure 1F**), suggesting that IFN-β is involved in I/R-induced myocardial injury. Overall, these results suggest that IFN-β strengthens I/R-induced myocardial injury.

LncRNA BANCR is involved in IFN-β-induced cardiomyocyte apoptosis

Subsequently, to investigate whether lncRNAs are involved in IFN-β-induced cardiomyocyte apoptosis, RNA-seq analysis was performed with normal cardiomyocytes and IFN-β-treated cardiomyocytes. The results revealed that 116 lncRNAs in IFN-β-treated cardiomyocytes were upregulated, and 57 lncRNAs were downregulated compared with normal cardiomyocytes (**Figure 2A**). In addition, these differentially expressed lncRNAs were mainly divided into four subgroups: lncRNAs (51.27%), anti-sense lncRNAs (3.78%), intronic lncRNAs (34.19%) and sense lncRNAs (10.76%) (**Figure 2B**). Subsequently, the basal expression levels of the ten most upregulated lncRNAs in normal cardiomyocytes were measured. As presented in **Figure 2C**, lncRNA-AK03516, MALAT1, SAP30L-AS1, OIP5-AS1 and BANCR were found to be highly expressed in normal cardiomyocytes, while GASL1, CTB-171A8.1, SNHG1, MEG3 and TUG1 were expressed at relatively lower levels. Given that lncRNA BANCR has been reported to be induced by IFN-γ in retinal pigment epithelial cells, and promotes apoptosis of retinal pigment epithelial cells [27, 28], the present study selected BANCR for further investigation. Subsequently, BANCR expression levels were detected in different types of cells, and it was identified that BANCR is highly expressed in cardiomyocytes and macrophages, and expressed at relatively lower levels in endothelial cells, $CD4^+$ T cells, and fibroblasts (**Figure 2D**). Furthermore, IFN-β stimulation significantly increased BANCR expression in cardiomyocytes and macrophages, but not in endothelial cells, $CD4^+$ T cells, and fibroblasts (**Figure 2E**), suggesting BANCR may participate in regulating immune responses.

Subsequently, it was investigated whether lncRNA BANCR was related to IFN-β-induced cardiomyocyte apoptosis. As shown in **Figure 2F** and **2G**, BANCR-knockdown by transfection with specific siRNA evidently suppressed IFN-β-induced cardiomyocyte apoptosis. In addition, pretreatment with anti-IFNAR1 antibody significantly suppressed IFN-β-triggered myocardial apoptosis, whereas BANCR-knockdown did not further augment the effects of anti-IFNAR1 antibody on IFN-β-triggered myocardial apoptosis (**Figure 2H**), suggesting BANCR may act downstream of IFN-IFNAR1 signaling to promote IFN-β-induced myocardial apoptosis. Taken together, these results suggest that lncRNA BANCR is involved in IFN-β-induced cardiomyocyte apoptosis.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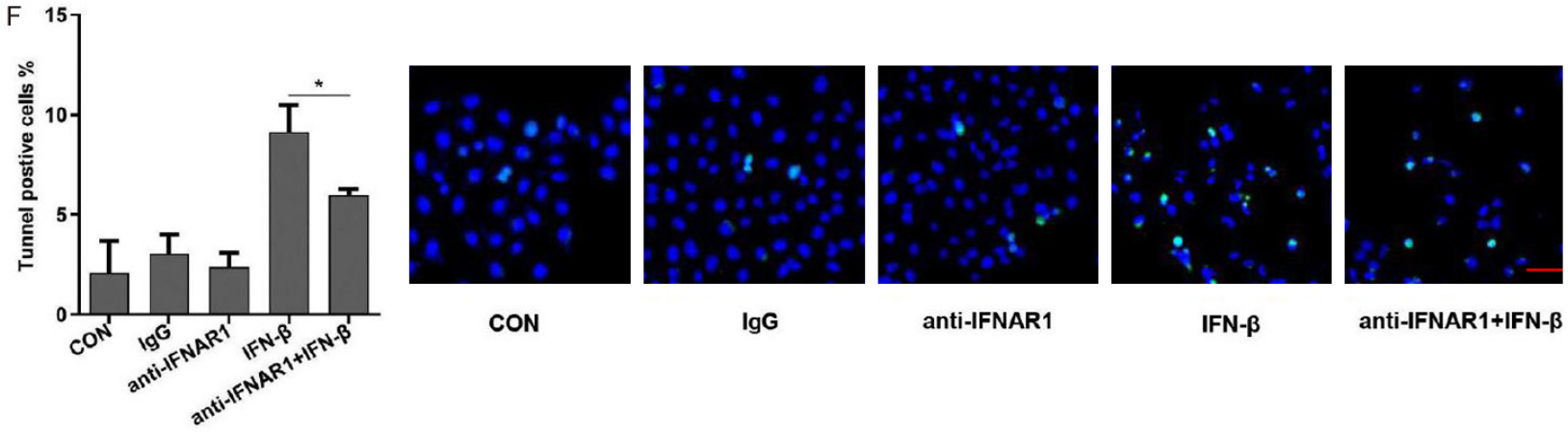


Figure 1. IFN-β, which is induced by I/R in cardiomyocytes, increases I/R-induced myocardial injury. iPS cell-originated cardiomyocytes were treated with oxygen and glucose deprivation for 12 h followed by reperfusion for 4, 8 or 12 h to mimic I/R injury, and then cellular apoptosis was detected by (A) TUNEL assay and (B) IFN-β expression in the culture supernatants was detected by ELISA. Normal cardiomyocytes were used as CON (B). (C) Cardiomyocytes were pretreated with anti-IFNAR1 antibody or not for 2 h, and then treated with I/R for the indicated times. Cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. (D) Cardiomyocytes were treated with IFN-β or PBS for the indicated times, and then cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. (E) Cardiomyocytes were treated with IFN-β and I/R for the indicated times, and then cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. (F) Cardiomyocytes were treated with anti-IFNAR1 antibody, control IgG (Ab), IFN-β, or anti-IFNAR1 antibody and IFN-β for 12 h, and then cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. Data are presented as mean ± SD (n=3). *P<0.05. IFN, interferon; I/R, ischemia-reperfusion; CON, control; IFNAR1, IFN α/β receptor 1 antibody; ELISA, enzyme-linked immunosorbent assay.

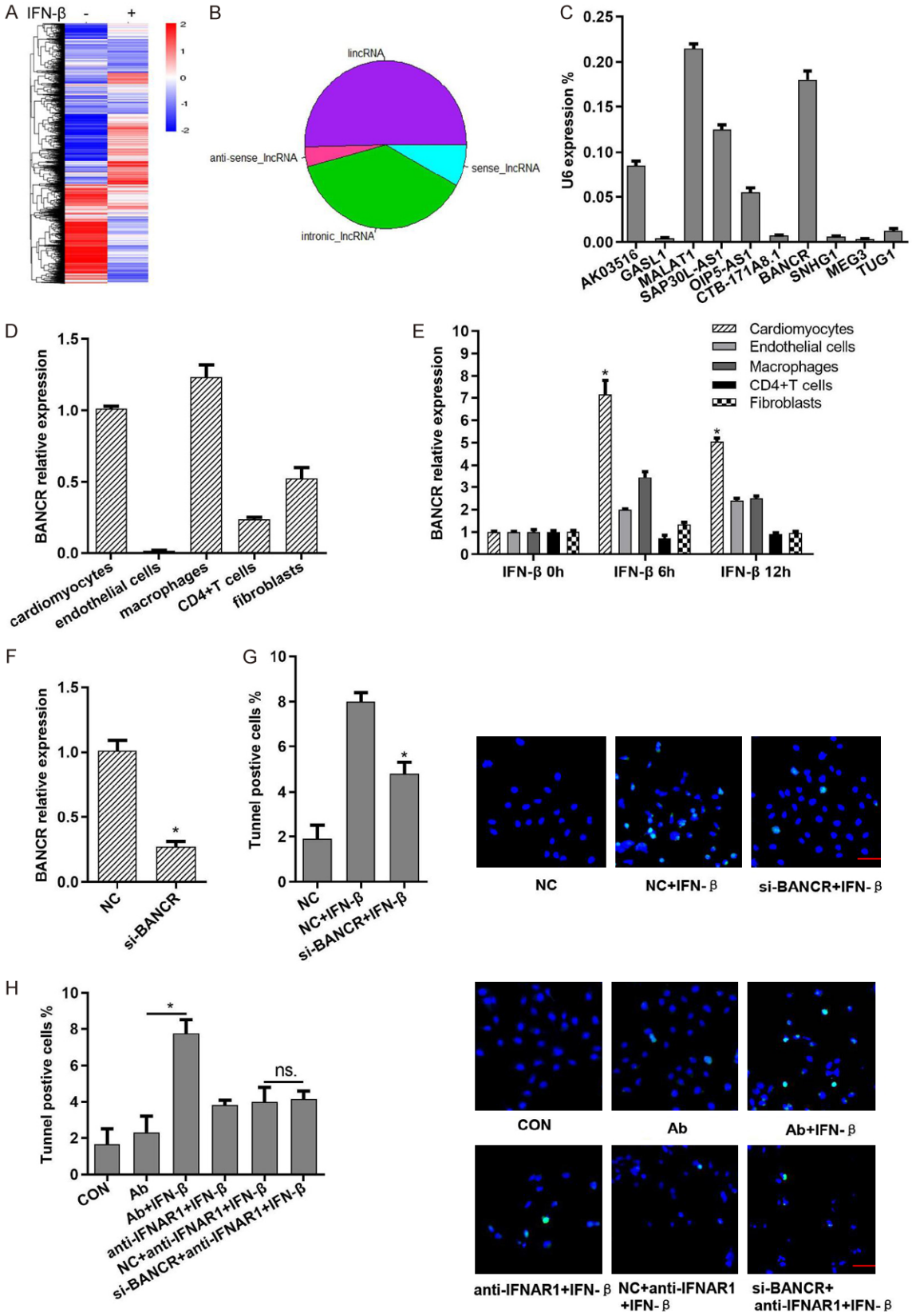


Figure 2. LncRNA BANCR is involved in IFN-β-induced cardiomyocyte apoptosis. A. Heat map of lncRNAs in cardiomyocytes treated with IFN-β for 12 h or not. B. Pie chart showing the percentage distribution of different subgroups

of lncRNAs. C. Relative expression levels of the indicated lncRNAs in cardiomyocytes were detected by RT-qPCR. D. Relative expression levels of lncRNA BANCR in the indicated cells were detected by RT-qPCR. E. Cells were treated with IFN-β for the indicated times, and then relative expression levels of lncRNA BANCR were detected by RT-qPCR. F. Cardiomyocytes were transfected with si-BANCR or control siRNA for 24 h, and then relative expression levels of lncRNA BANCR were detected by RT-qPCR. G. Cardiomyocytes were transfected with si-BANCR or control siRNA for 24 h, and then treated with IFN-β for 12 h. Cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. H. Cardiomyocytes were transfected with si-BANCR or control siRNA for 24 h, treated with anti-IFNAR1 antibody or control IgG (Ab) for 2 h, and then treated with IFN-β for 12 h. Cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. Data are presented as means ± SD (n=3). *P<0.05. NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; lncRNA, long non-coding RNA; BANCR, BRAF-activated non-coding RNA; IFN, interferon; si, small interfering; IFNAR1, IFN α/β receptor 1 antibody.

LncRNA BANCR-induced promotional effect on IFN-β-induced cardiomyocyte apoptosis depends on STAT1

Subsequently, we examined the effects of lncRNA BANCR on downstream of the IFN-IFNAR1 signaling in cardiomyocytes. As presented in **Figure 3A**, IFN-β treatment significantly induced the phosphorylation of STAT1 and STAT2, whereas BANCR-knockdown evidently inhibited IFN-β-induced phosphorylation of STAT1, but not STAT2. Furthermore, BANCR-knockdown significantly inhibited IFN-β-induced pro-apoptotic ISG expression, including CD95, TNF-α and NOS2 in cardiomyocytes (**Figure 3B**). Consistently, BANCR-overexpression mediated by AAV infection significantly enhanced IFN-β-induced STAT1 phosphorylation and promoted CD95, TNF-α, and NOS2 expression levels (**Figure 3C-E**). Furthermore, BANCR-overexpression further augmented IFN-β-induced cardiomyocyte apoptosis (**Figure 3F**). Additionally, it was investigated whether the lncRNA BANCR-induced promotion of IFN-β-induced cardiomyocyte apoptosis was associated with STAT1. The results demonstrated that transfection with siRNA against BANCR or treatment with a specific inhibitor of STAT1, fludarabine, significantly suppressed IFN-β-induced cardiomyocyte apoptosis. Overall, these results indicate that the lncRNA BANCR-induced promotion of IFN-β-induced cardiomyocyte apoptosis depends on STAT1.

LncRNA BANCR promotes IFN-β-induced cardiomyocyte apoptosis by interacting with STAT1

To further investigate the underlying molecular mechanisms of the lncRNA BANCR-induced promotion of IFN-β-induced cardiomyocyte apoptosis, the present study performed RNA pull-down with transcribed biotinylated lncRNA BANCR *in vitro* accompanied by mass spectrometry using nuclear extracts of IFN-β-treated cardiomyocytes to identify the lncRNA BANCR-interacting proteins in cardiomyocytes (**Figure 4A** and **4B**). Among the identified BANCR-interacting proteins in cardiomyocytes (**Figure 4C**), the current study focused on STAT1, due to the fact that BANCR-overexpression significantly enhanced IFN-β-induced STAT1 phosphorylation in cardiomyocytes (**Figure 3D**). Furthermore, RIP analysis demonstrated that STAT1 indeed interacted with BANCR in cardiomyocytes (**Figure 4D**). In addition, RNA pull-down analysis also demonstrated that BANCR interacted with STAT1 in cardiomyocytes (**Figure 4E**). Moreover, IFN-β treatment promoted the interaction of STAT1 with BANCR in cardiomyocytes (**Figure 4F**). In addition, BANCR-overexpression significantly promoted IFN-β-induced nuclear location of STAT1 (**Figure 4G**). Overall, these results indicate that BANCR promotes IFN-β-induced cardiomyocyte apoptosis by interacting with STAT1.

Discussion

I/R-induced cardiomyocyte apoptosis contributes to heart failure, rupture or dysfunction following the occurrence of acute myocardial infarction [29]. Recent studies have reported that the myocardium functions as an immune organ, and I/R initiates myocardial innate immune responses, which fuels a fatal response to myocardial infarction [10-12]. Due to the high basal expression of IFN-β in cardiomyocytes, it was hypothesized that basal secreted IFN-β participates in I/R-induced cardiomyocyte apoptosis. Our confirmed this hypothesis and found that I/R significantly induces IFN-β expression in iPS cell-derived cardiomyocytes, and IFN-β further strengthens I/R-induced myocardial apoptosis. Subsequently, the current study further investigated the role of lncRNAs during IFN-β-induced cardiomyocyte apoptosis and found that lncRNA BANCR promotes

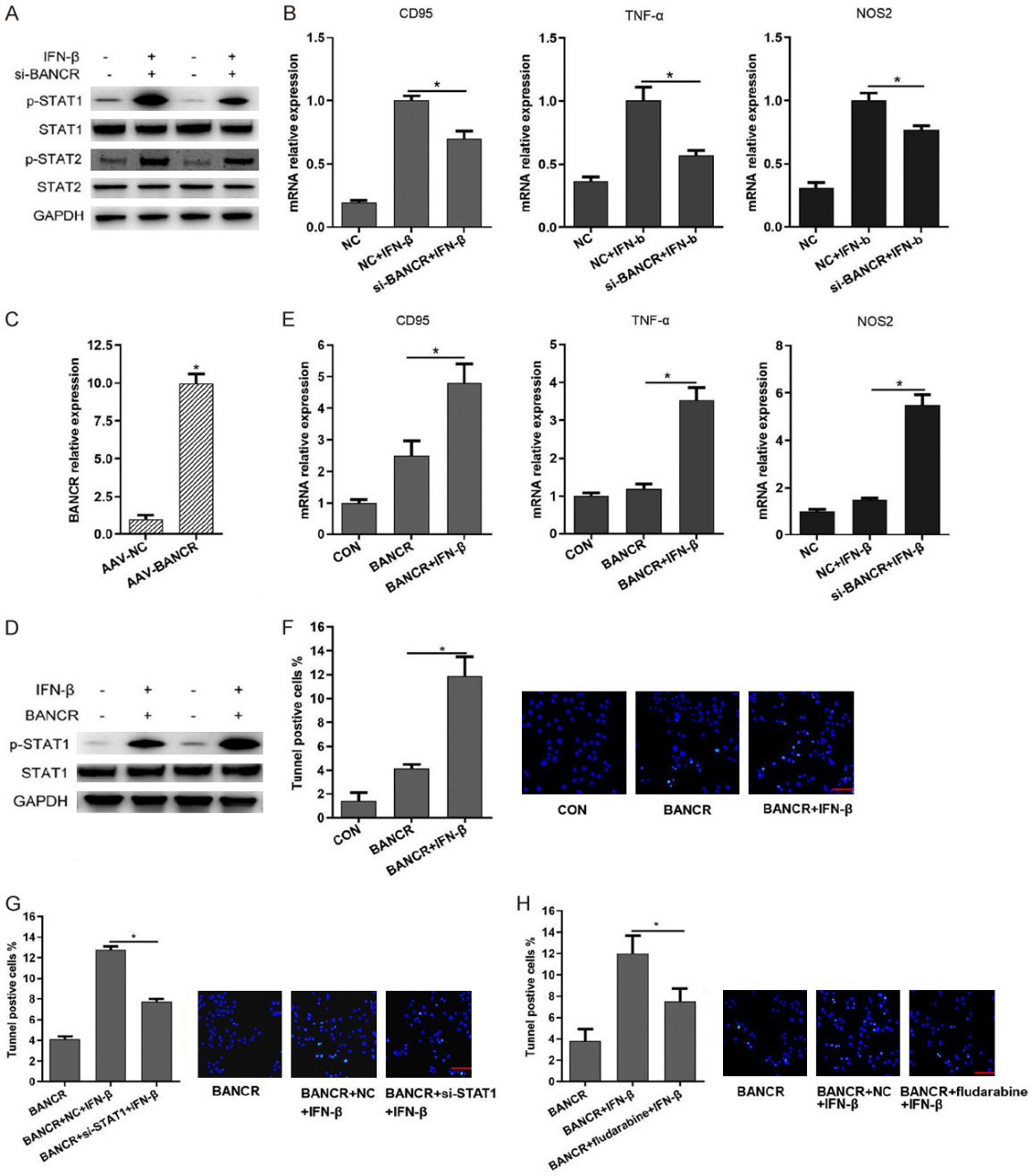
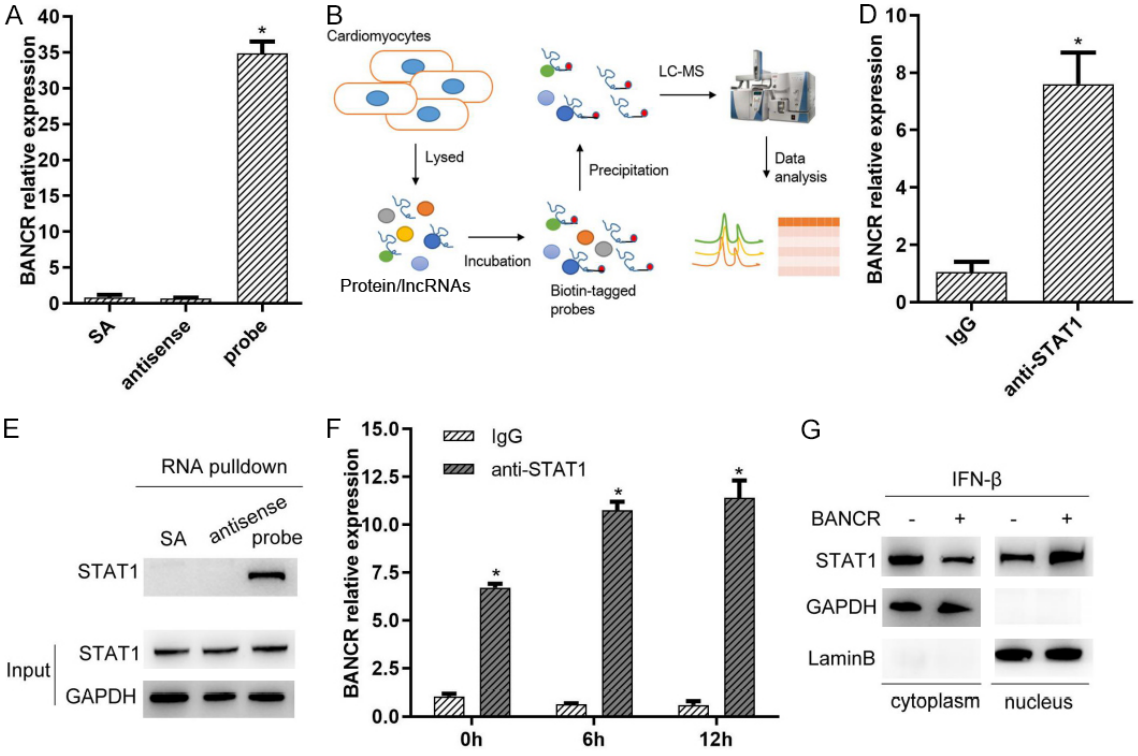


Figure 3. lncRNA BANCR-induced promotion of IFN-β-induced cardiomyocyte apoptosis depends on STAT1. A. Cardiomyocytes were transfected with si-BANCR or control siRNA for 24 h, and then treated with IFN-β or not for 1 h. p-STAT1, STAT1, p-STAT2, STAT2 and GAPDH protein expression levels were detected using western blotting. B. Cardiomyocytes were transfected with si-BANCR or control siRNA for 24 h, and then treated with IFN-β or not for 12 h. Relative mRNA expression levels of CD95, TNF-α and NOS2 were detected by RT-qPCR. C. Cardiomyocytes were infected with AAV-NC or AAV-BANCR for 24 h, and then relative mRNA expression levels of BANCR were detected by RT-qPCR. Cardiomyocytes were infected with AAV-NC or AAV-BANCR for 24 h, and then treated with IFN-β for 1 h or 12 h. D. p-STAT1, STAT1, p-STAT2, STAT2 and GAPDH protein expression levels were detected using western blotting. E. Relative mRNA expression levels of CD95, TNF-α, and NOS2 were detected by RT-qPCR. F. Cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. G. Cardiomyocytes were infected with AAV-BANCR for 24 h, transfected with si-BANCR or control siRNA for 24 h, and then treated with IFN-β or not for 12 h. Cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. H. Cardiomyocytes were infected with AAV-BANCR for 24 h, treated with the STAT1 activation inhibitor, fludarabine, for 2 h, and then treated with IFN-β or not for 12 h. Cellular apoptosis was detected by TUNEL assay. Scale bar, 40 μM. Data are presented as means ± SD (n=3). *P<0.05. RT-qPCR, reverse transcription-quantitative PCR; lncRNA, long non-coding RNA; BANCR, BRAF-activated non-coding RNA; IFN, interferon; si, small interfering; p-, phosphorylated; STAT, signal transducer and activator of transcription.



C

The top20 identified Proteins potentially interacting with BANCR

Gene Symbol	Protein	Count	Length	fscore
HMX1	HMX1	6	348	0.99
CE024	C5orf24	5	188	0.99
TE2IP	TERF2IP	15	399	0.98
THYN1	THYN1	6	225	0.98
HXB9	HOXB9	6	250	0.97
3MG	MPG	10	298	0.96
KAD2	AK2	5	239	0.96
NFIC	NFIC	5	508	0.96
TFAM	TFAM	11	246	0.95
TPX2	TPX2	10	747	0.95
NFIX	NFIX	5	502	0.95
AURKB	AURKB	5	344	0.94
RCC2	RCC2	18	522	0.93
STAT1	STAT1	23	750	0.91
CFTR	CFTR	5	421	0.91
DNLI4	LIG4	6	911	0.9
SSRA	SSR1	8	286	0.88

Figure 4. lncRNA BANCR promotes IFN-β-induced cardiomyocyte apoptosis by interacting with STAT1. A. BANCR abundance was examined in cellular extracts from cardiomyocytes with biotin-labeled BANCR-pull-down by RT-qPCR. Anti-sense BANCR or SA dynabeads was used as the control. B. A diagram for RNA pull-down accompanied by mass spectrometry. C. The top 20 proteins that may interact with BANCR in cardiomyocytes were identified by mass spectrometry. D. The interaction between STAT1 and BANCR was detected by RIP. E. The interaction between BANCR and STAT1 was detected by RNA pull-down. F. The interaction between STAT1 and BANCR in IFN-β treated-cardiomyocytes was detected by RIP. G. Cardiomyocytes were infected with AAV-BANCR for 24 h, and then treated with IFN-β or not for 1 h. STAT1 expression in the cytoplasm or nucleus was detected by western blotting. Data are presented as means ± SD (n=3). *P<0.05. SA, streptavidin; RT-qPCR, reverse transcription-quantitative PCR; lncRNA, long non-coding RNA; BANCR, BRAF-activated non-coding RNA; IFN, interferon; RIP, RNA-binding protein immunoprecipitation; STAT, signal transducer and activator of transcription.

IFN-β-induced cardiomyocyte apoptosis after I/R injury by interacting with STAT1.

There are also some limitations of the present study. First, the study mainly focused on IFN-β-induced cardiomyocyte apoptosis, which may be a partial underlying mechanism for causing cardiomyocyte apoptosis following myocardial I/R. However, high basal IFN-β expression in cardiomyocytes indeed aggravates I/R-induced cardiomyocyte apoptosis. Due to the complexity of the cellular composition in the heart, there are also some macrophages and fibroblasts, and little lymphocytes in heart tissues [30-32]. A previous study demonstrated that I/R-induced cardiomyocyte-derived nucleic acid materials elicit IFN-related inflammation of macrophages, which promotes ventricular dilation and aggravates cardiac function *in vivo* [11]. The present study only investigated the role of cardiomyocyte-derived IFN-β. When acute myocardial infarction occurs, IFN-β expression may arise more sharply and could be secreted not only by cardiomyocytes but also by macrophages, fibroblasts, lymphocytes, or other cell types. Therefore, it can be speculated that IFN-β-induced cardiomyocyte apoptosis may be more prominent in an animal model of myocardial I/R. Regarding the mechanism, lncRNA BANCR interacts with STAT1 to promote STAT1 phosphorylation, resulting in enhanced pro-apoptotic ISG production. Moreover, anti-inflammatory interleukin-10 (IL-10) is also induced by JAK/STAT signaling, and negatively regulates expressions of TH1 cytokines and MHC class II antigens on macrophages [33], Whether BANCR also influences interleukin-10 expression through JAK/STAT1 signaling needs to be explored in the following studies. In addition, other functions of BANCR during cardiomyocyte apoptosis remain unknown. Focusing on the changes of lncRNA alone during IFN-β-induced STAT1 phosphorylation may be a limitation of the present study, as there are also other factors involved, such as tyrosine kinase 2, suppressor of cytokine signaling 1/3, and heat shock protein 90 [34-36]. Determining the protein interactions of STAT1 during cardiomyocyte apoptosis may also be important for the development of therapeutic targets.

In addition to STAT1, some other proteins that potentially interact with lncRNA BANCR in cardiomyocytes were identified by MS, such as mitochondrial adenylate kinase 2 (AK2) and homeobox B9. Energy dysfunction is closely associated with heart failure, and AK2 catalyzes the reversible reaction of 2ADP (GDP) ↔ ATP (GTP) + AMP, to effect cellular energy homeostasis [37]. With an apoptotic stimulus, AK2 translocates from mitochondria into the cytosol, and then induces apoptosis by formation of an AK2-FADD-caspase-10 complex [38]. Furthermore, AK2 is a regulator of cardiogenesis and heart tissue regeneration [39]. Whether AK2 is involved in LncRNA BANCR regulating IFN-β induced cardiomyocyte apoptosis needs to be explored in the following studies.

In conclusion, our study reports for the first time that LncRNA BANCR expression promotes IFN-β-mediated cardiomyocyte apoptosis after ischemia-reperfusion injury by interacting with STAT1.

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Disclosure of conflict of interest

None.

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References

[1] Hausenloy DJ and Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. J Clin Invest 2013; 123: 92-100.
[2] Kuznetsov AV, Javadov S, Margreiter R, Grimm M, Hagenbuchner J and Ausserlechner MJ. The role of mitochondria in the mechanisms of cardiac ischemia-reperfusion injury. Antioxidants (Basel) 2019; 8: 454.
[3] Yao L, Chen H, Wu Q and Xie K. Hydrogen-rich saline alleviates inflammation and apoptosis in myocardial I/R injury via PINK-mediated autophagy. Int J Mol Med 2019; 44: 1048-62.
[4] Tang L, Mo Y, Li Y, Zhong Y, He S, Zhang Y, Tang Y, Fu S, Wang X and Chen A. Urolithin a alleviates myocardial ischemia/reperfusion injury via PI3K/Akt pathway. Biochem Biophys Res Commun 2017; 486: 774-80.
[5] Mo D, Tian W, Zhang HN, Feng YD, Sun Y, Quan W, Hao XW, Wang XY, Liu XX, Li C, Cao W, Liu WJ

and Li XQ. Cardioprotective effects of galectin-3 inhibition against ischemia/reperfusion injury. Eur J Pharmacol 2019; 863: 172701.
[6] Goubau D, Deddouche S and Reis e Sousa C. Cytosolic sensing of viruses. Immunity 2013; 38: 855-69.
[7] Woo SR, Fuertes MB, Corrales L, Spranger S, Furdyna MJ, Leung MY, Duggan R, Wang Y, Barber GN, Fitzgerald KA, Alegre ML and Gajewski TF. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. Immunity 2014; 41: 830-42.
[8] Gall A, Treuting P, Elkon KB, Loo YM, Gale M Jr, Barber GN and Stetson DB. Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease. Immunity 2012; 36: 120-31.
[9] Wu J, Sun L, Chen X, Du F, Shi H, Chen C and Chen ZJ. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science 2013; 339: 826-30.
[10] Vilahur G and Badimon L. Ischemia/reperfusion activates myocardial innate immune response: the key role of the toll-like receptor. Front Physiol 2014; 5: 496.
[11] King KR, Aguirre AD, Ye YX, Sun Y, Roh JD, Ng RP Jr, Kohler RH, Arlauckas SP, Iwamoto Y, Savol A, Sadreyev RI, Kelly M, Fitzgibbons TP, Fitzgerald KA, Mitchison T, Libby P, Nahrendorf M and Weissleder R. IRF3 and type I interferons fuel a fatal response to myocardial infarction. Nat Med 2017; 23: 1481-87.
[12] Khan Faisal AW, Nisar S, Ali SA and Ahmad F. Effects of interferon therapy on heart. J Ayub Med Coll Abbottabad 2016; 28: 276-80.
[13] Li X, Luo S, Zhang J, Yuan Y, Jiang W, Zhu H, Ding X, Zhan L, Wu H, Xie Y, Song R, Pan Z and Lu Y. lncRNA H19 alleviated myocardial I/RI via suppressing miR-877-3p/Bcl-2-mediated mitochondrial apoptosis. Mol Ther Nucleic Acids 2019; 17: 297-309.
[14] Rinn JL and Chang HY. Genome regulation by long noncoding RNAs. Annu Rev Biochem 2012; 81: 145-66.
[15] Zhang XC, Gu AP, Zheng CY, Li YB, Liang HF, Wang HJ, Tang XL, Bai XX and Cai J. YY1/LncRNA GAS5 complex aggravates cerebral ischemia/reperfusion injury through enhancing neuronal glycolysis. Neuropharmacology 2019; 158: 107682.
[16] Archer K, Broskova Z, Bayoumi AS, Teoh JP, Davila A, Tang Y, Su H and Kim IM. Long non-coding RNAs as master regulators in cardiovascular diseases. Int J Mol Sci 2015; 16: 23651-67.
[17] Jung M, Dodsworth M and Thum T. Inflammatory cells and their non-coding RNAs as targets for treating myocardial infarction. Basic Res Cardiol 2018; 114: 4.
[18] Zhang BF, Chen J and Jiang H. LncRNA H19 ameliorates myocardial ischemia-reperfusion injury by targeting miR-22-3P. Int J Cardiol 2019; 278: 224.
[19] Wang SM, Liu GQ, Xian HB, Si JL, Qi SX and Yu YP. LncRNA NEAT1 alleviates sepsis-induced myocardial injury by regulating the TLR2/NF-kappaB signaling pathway. Eur Rev Med Pharmacol Sci 2019; 23: 4898-907.
[20] Li H, Liu X, Zhang L and Li X. LncRNA BANCR facilitates vascular smooth muscle cell proliferation and migration through JNK pathway. Oncotarget 2017; 8: 114568-75.
[21] Shiba Y, Gomibuchi T, Seto T, Wada Y, Ichimura H, Tanaka Y, Ogasawara T, Okada K, Shiba N, Sakamoto K, Ido D, Shiina T, Ohkura M, Nakai J, Uno N, Kazuki Y, Oshimura M, Minami I and Ikeda U. Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. Nature 2016; 538: 388-91.
[22] Satsuka A and Kanda Y. Cardiotoxicity assessment of drugs using human iPS cell-derived cardiomyocytes: from proarrhythmia risk to cardiooncology. Curr Pharm Biotechnol 2020; 21: 765-772.
[23] Zhang W, Li Y and Wang P. Long non-coding RNA-ROR aggravates myocardial ischemia/reperfusion injury. Braz J Med Biol Res 2018; 51: e6555.
[24] Pike KA, Hutchins AP, Vinette V, Theberge JF, Sabbagh L, Tremblay ML and Miranda-Saavedra D. Protein tyrosine phosphatase 1B is a regulator of the interleukin-10-induced transcriptional program in macrophages. Sci Signal 2014; 7: ra43.
[25] Wang S, He F, Li Z, Hu Y, Huangfu N and Chen X. YB1 protects cardiac myocytes against H2O2 induced injury via suppression of PIAS3 mRNA and phosphorylation of STAT3. Mol Med Rep 2019; 19: 4579-88.
[26] Bao X, Wu H, Zhu X, Guo X, Hutchins AP, Luo Z, Song H, Chen Y, Lai K, Yin M, Xu L, Zhou L, Chen J, Wang D, Qin B, Frampton J, Tse HF, Pei D, Wang H, Zhang B and Esteban MA. The p53-induced lincRNA-p21 derails somatic cell reprogramming by sustaining H3K9me3 and CpG methylation at pluripotency gene promoters. Cell Res 2015; 25: 80-92.
[27] Kutty RK, Samuel W, Duncan T, Postnikova O, Jaworski C, Nagineni CN and Redmond TM. Proinflammatory cytokine interferon-gamma increases the expression of BANCR, a long non-coding RNA, in retinal pigment epithelial cells. Cytokine 2018; 104: 147-50.
[28] Yin L, Sun Z, Ren Q, Su X and Zhang D. Long non-coding RNA BANCR is overexpressed in patients with diabetic retinopathy and promotes apoptosis of retinal pigment epithelial cells. Med Sci Monit 2019; 25: 2845-51.

[29] Lu L, Liu M, Sun R, Zheng Y and Zhang P. Myocardial infarction: symptoms and treatments. Cell Biochem Biophys 2015; 72: 865-7.
[30] Lavine KJ, Epelman S, Uchida K, Weber KJ, Nichols CG, Schilling JD, Ornitz DM, Randolph GJ and Mann DL. Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. Proc Natl Acad Sci U S A 2014; 111: 16029-34.
[31] Liu B, Zhang HG, Zhu Y, Jiang YH, Luo GP, Tang FQ, Jian Z and Xiao YB. Cardiac resident macrophages are involved in hypoxiainduced postnatal cardiomyocyte proliferation. Mol Med Rep 2017; 15: 3541-48.
[32] Yang W, Zhang S, Zhu J, Jiang H, Jia D, Ou T, Qi Z, Zou Y, Qian J, Sun A and Ge J. Gut microbe-derived metabolite trimethylamine N-oxide accelerates fibroblast-myofibroblast differentiation and induces cardiac fibrosis. J Mol Cell Cardiol 2019; 134: 119-30.
[33] Kadokami T, McTiernan CF, Kubota T, Frye CS, Bounoutas GS, Robbins PD, Watkins SC and Feldman AM. Effects of soluble TNF receptor treatment on lipopolysaccharide-induced myocardial cytokine expression. Am J Physiol Heart Circ Physiol 2001; 280: H2281-91.
[34] Woss K, Simonovic N, Strobl B, Macho-Maschler S and Muller M. TYK2: an upstream kinase of STATs in cancer. Cancers (Basel) 2019; 11: 1728.
[35] Khan MGM, Ghosh A, Variya B, Santharam MA, Kandhi R, Ramanathan S and Ilangumaran S. Hepatocyte growth control by SOCS1 and SOCS3. Cytokine 2019; 121: 154733.
[36] Mahendrarajah N, Borisova ME, Reichardt S, Godmann M, Sellmer A, Mahboobi S, Haitel A, Schmid K, Kenner L, Heinzel T, Beli P and Kramer OH. HSP90 is necessary for the ACK1-dependent phosphorylation of STAT1 and STAT3. Cell Signal 2017; 39: 9-17.
[37] Chen RP, Liu CY, Shao HL, Zheng WW, Wang JX and Zhao XF. Adenylate kinase 2 (AK2) promotes cell proliferation in insect development. BMC Mol Biol 2012; 13: 31.
[38] Kohler C, Gahm A, Noma T, Nakazawa A, Orrenius S and Zhivotovsky B. Release of adenylate kinase 2 from the mitochondrial intermembrane space during apoptosis. FEBS Lett 1999; 447: 10-2.
[39] Dzeja PP, Chung S, Faustino RS, Behfar A and Terzic A. Developmental enhancement of adenylate kinase-AMPK metabolic signaling axis supports stem cell cardiac differentiation. PLoS One 2011; 6: e19300.