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# Long Noncoding RNAs: Important Participants and Potential Therapeutic Targets for Myocardial Ischaemia Reperfusion Injury

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**Short title: LncRNAs in Myocardial Ischaemia Reperfusion**

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# Long Noncoding RNAs: Important Participants and Potential Therapeutic Targets for Myocardial Ischaemia Reperfusion Injury

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**Abstract**

Myocardial ischaemia reperfusion (I/R) injury is one of the leading causes of coronary artery disease-associated morbidity and mortality. While different strategies have been used to limit I/R injuries, cardiac functions often do not recover tothe normal level as anticipated. Recent studies have pointed to important roles of long non-coding RNAs (lncRNAs) in the development of myocardial I/R injury. LncRNA is a class of RNA molecules of more than 200 nucleotides in length whichare not translated into proteins. I/R causes dysregulation of lncRNA expression in cardiomyocytes, thereby affecting multiple cellular functions including mitochondrial homeostasis, apoptosis, necrosis and autophagy, suggesting that manipulating lncRNAs may be of great potentialsin counteracting I/R injury-induced myocardial dysfunctions.In this review, we provide an updated summary on our knowledge about contributions of lncRNAs to the development of I/R injury, with an emphasis on the functional links between several well established cardiac lncRNAs and regulation of cellular outcomes post I/R.

**KEYWORDS:**

apoptosis; autophagy; cardiomyocyte; ischaemia and reperfusion injury; long non-coding RNA; LncRNA; necroptotic cell death

## 1. Introduction

The incidence of coronary artery disease has been on the rise worldwide, which has a significant contribution to the total deaths caused by cardiovascular disorders[1,2]. Restoring blood flow (reperfusion) to the ischaemic myocardium by coronary artery bypass grafting, percutaneous coronary intervention, or thrombolytic therapy is currently the frontline treatment method. However, functional recovery of the ischaemic myocardium is not always optimal after these interventions, mainly due to the occurrence of ischaemiareperfusion (I/R) injury[3,4]. The pathophysiological mechanism of I/R injury centres on intracellular calcium overload and reactive oxygen species production, leading to various organelle dysfunctions (especially loss of mitochondrial homeostasis)[3,4]. I/R injury may cause cell death in different manners, including apoptosis, necrosis, necroptotic cell death, and arguably autophagy-related cell death[5]. Clinically, I/R injury is associated with declined cardiac functions and worsenedprognosis in patients with ischaemic heart disease, and is a major factor limiting the effectiveness of reperfusion therapies. As such, there is still an urgent need for better treatment strategies to reduce the impactsof I/R injury.

Long non-coding RNA (lncRNA) refers to a class of RNA molecules of more than 200 nucleotides in length which are not translated to proteins[6-8]. These special RNA molecules are produced via transcription from intergenic regions of the genome, from within introns of protein-coding genes, or from the antisense strand of a given gene. Mounting evidence supports the notion that LncRNAs may have important roles in regulatinggene expressions, and their functions can be either stimulatory or inhibitory[6-8]. The principal

molecular mechanisms by which LncRNAs regulate gene expressions include the following ones: (1)LncRNAs may bind to conventional transcription factors or chromatin-modifying enzymes (such aspolycomb1repressive complex, histone demethylases, and DNA methyltransferases)[9]. This in turn can result in different circumstances. On one hand, lncRNAs may guide the protein factors to the target DNA thereby silencing or activating the transcription of relevant genes, whereas on the other the double stranded regions of lncRNAs can mimic transcription factor binding sites, thereby functioning as a decoy to preventtranscription factors from binding to their targets. (2) LncRNAs can either bind to exon/intron junctions in pre–mRNAs, act as a scaffold in ribonucleoprotein complexes, or mask binding sites for proteins or miRNAs within the mRNA strand, thereby modulating pre-mRNA alternative splicing and mRNA stability. (3) LncRNAs are able to bind microRNAs (miRNAs), in that way indirectly modulategene expression by preventing the miRNAs from binding to and regulating their mRNA targets; this mode of action is termed "competing endogenous RNAs (ceRNAs)" or "miRNA sponges".[6-8](see Figure 1).

Recently, different lines ofstudies have identified that cardiac I/R injury is associated with alterations in lncRNA expressions. Further molecular mechanistic studies in animal models have indicated that lncRNAs may have both protective and detrimental roles in the pathogenesis of I/R injury, especially in modulating cell death. In this review, we aim to provide an updated summary on our knowledge about contributions of lncRNAsto the development of I/R injury. From a pathophysiological point of view, we focus on the functional links between several well established cardiac lncRNAs and regulation of cellular outcomes post I/R. Potential application of lncRNAsas biomarkers in patients with ischaemic heart disease will also be discussed.

## 2. Dysregulation of LncRNA Expressions Following Myocardial I/R Injury

Studies in rodent models have shown that I/R causes dysregulated lncRNAexpressions in cardiactissue[10-12].For instance,Liu et al.have found that 151 lncRNAs are differentially expressed (with 64 upregulated and 87 downregulated) in I/R-treated myocardium from rats[12]. Lin et al. have compared the expression of both lncRNAs and mRNAs in rat myocardium with I/R injury to sham controls[10], and identified upregulations in 851 lncRNAs and 1,015 mRNAs, together with downregulations in 1,533 lncRNAs and 1,702 mRNAs. Among these dysregulated lncRNAs, 12 have been confirmed to be related to genes encoding for apoptosis-related proteins such as STAT3 and IL1R1[10]. Huang et al.have explored the effect of I/R on lncRNA expressions inH9c2 myocytes and Langendorff-perfused hearts from rats, showing that 797 lncRNAs and 1898 mRNAs are differentially expressed afterI/R challenge[11], with a similar trend ofchanges observed inboth of the cell culture and the cardiac tissue. Collectively, these results support the notion that I/R causes a dysregulation of the expression of cardiac lncRNAs.

## 3. Regulation of Cellular Outcomes by LncRNAs and Potential Molecular Mechanisms in Myocardial I/R Injury

### 3.1 LncRNAswithAnti-ApoptoticEffects

Urothelial carcinoma-associated 1 (UCA1) is first cloned from human bladder transitional cell carcinoma cell line BLZ-211[13], which regulates genes that are involved in tumorigenic functions including apoptosis, pre-mRNA splicing, cell cycle and extracellular matrix remodelling[13]. Based on these findings, UCA1 has been explored for its involvementin regulating apoptosis during myocardial I/Rinjury[14,15].Increased production of $H_2O_2$ during I/R downregulates UCA1, and this consequently increases p27 expression therebypromotingmyocyte apoptosis[14,15]. Moreover, UCA1 has also been implicated in morphine-induced post-conditioning protection, which limits I/R injury in the rat myocardiumthrough inhibitingmiR-128 expression[16].

FTX (Five prime to Xist) is a conserved lncRNA which is localized in the inactivation centre of X chromosome[17]. FTX is downregulated upon I/R injury, while enhanced expression of FTX inhibits cardiomyocyte apoptosis[18]. The anti-apoptotic effect of FTX is suggested to be through sponging miR-29b-1-5p and protecting the cell survival protein Bcl-2-like protein 2[18].

Evidence has suggested that modulating mitochondrial homeostasis may be one important mechanism by which LncRNA suppresses cell apoptosis. Fission and fusion are key dynamic processes that control the number, morphology and activity of mitochondria in a cell[19]. LncRNAs have been shown to participate in modulating mitochondrial fission in myocardial I/R injury[20,21]. For instance, CARL (cardiac apoptosis-related lncRNA) interferes with miR-539, which in turn targets prohibitin 2[20]. Found primarily in the mitochondrial inner membrane, prohibitin proteins assemble to form a supra-macromolecular structure, which works as a scaffold for proteins and lipids regulating mitochondrial metabolism, including bioenergetics, biogenesis, and dynamics[22]. Overexpression of prohibitin 2 in the myocardium limits infarct size in rats with myocardial I/R, with concomitant decreases in mitochondrial fission and cell apoptosis[20]. Hence, CARL can protect prohibitin 2 by suppressing the expression of miR-539, leading to reduced cell apoptosis in I/R-challenged myocardium[20]. MDRL (mitochondrial dynamics related lncRNA) is another lncRNA that can regulate mitochondrial fission in cardiomyocytes under hypoxic condition[21]. By acting as an endogenous sponge for miR-361, MDRL has been shown to prevent mitochondrial degradation and cell apoptosis in hypoxia-reoxygenation-treated cardiomyocytes[21].

HOTAIR (HOX transcript antisense intergenic RNA) is a lncRNA which is originally discovered in tumours. Li et al. have found that HOTAIR inhibition can exacerbate oxidative stress-induced H9c2 cell apoptosis, whereas HOTAIR overexpression protects myocytes[23]. The anti-apoptotic effect of HOTAIR is thought to be related to the miR-125/matrix metalloproteinases-2 axis[23]. On the other hand, Meng et al. have demonstrated another molecular mechanism by which HOTAIR inhibits I/R-induced apoptosis in H9c2 cells[24]. This mechanism involves activation of the cytoprotective kinase AMPKα (AMP-activated protein kinase-α) through the enhancer of zeste homolog 2/microRNA-451/calcium-binding protein 39 axis[24]

### 3.2 LncRNAs with Pro-Apoptotic Effects

Amost investigated lncRNA with pro-apoptotic actions is MALAT1 (metastasis-associated lung adenocarcinoma transcript 1), which is initially discovered in non-small cell lung cancer[25]. For instance, there is evidence showing that induction of MALAT1 expression during I/R contributes to myocyte apoptosis by downregulating the expression of miRNA-144-3p[26]. The induction of MALAT1 during I/R has also been shown to promote myocyte apoptosis by repressing the expression of miR-145[27]. Interestingly, Li et al. have shown that miR-145 can protect cardiomyocytes from $H_2O_2$-induced apoptosis through inhibiting the expression of Bnip3, an initiation factor of the mitochondrial apoptotic pathway in cardiomyocytes[28]. On the other hand, MALAT1 can also promote myocyte apoptosis by upregulatingthe expression of factorswithpro-apoptotic activities during I/R, such as β-catenin[29]and miR-122[30].However, the pro-apoptotic activity of MALAT1 appears to be tissue-specific, since it shows protective effects in other organs undergoing I/R, such as brain[31] and skeletal muscle[32].

Liu et al. have reported that the lncRNA GAS5 (growth arrest-specific transcript 5) is abundantly expressed in I/R-treated H9c2 myocytes where it promotes cell apoptosis by upregulating LAS1, a less characterized protein probably involved in regulating cell membrane fluidity and permeability[33]. Of note, another possible mechanism by which GAS5 stimulates myocyte apoptosis is by increasing the expression of ROCK1 (Rho-associated protein kinase 1), leading to the opening of mitochondrial permeability transition pore[34]. In addition, there is evidence indicating that GAS5 may interact with miR-532-5p, causing miR-532-5p downregulation and inactivation of the phosphoinositide-3-kinase /AKT pathway[35].

The lncRNA H19 is originally identified as an embryonic gene product[36]. Luo and colleagues have demonstrated that an inhibition of H19 attenuates myocardial I/R injury by limiting apoptosis, inflammation and oxidative stress in cardiac cells[37]. The detrimental effects of H19 in myocytes involve production of miR-675 (the mature gene product of H19), and downregulation of the expression of peroxisome proliferator-activated receptor-α[37].

### *3.3 LncRNAs Involved in Modulating Necroptotic Cell Death*

A large number of cardiac cells undergo necrosis during I/R, which is traditionally thought to be an unregulated form of cell death. In recent years, evidence has suggested that a type of programmed necrotic cell death, termed necroptosis, exists in cells and may contribute to the loss of cardiac myocytes during heart failure[38].Morphologically, necroptosis resemblesin some aspects the process of conventional necrosis as well as maladaptive autophagy[38]. At the molecular level, necroptosis is mediated through receptor-interacting protein kinase 1 and 3 (RIP1, RIP3)and mixed lineage kinase domain-like protein[38].

In primary murine cardiomyocytes, the lncRNA NRF (necrosis related factor) is significantly upregulated by $H_2O_2$[39]. NRF binds to miR-873 and represses miR-873 expression as an endogenous microRNA sponge. On the other hand, miR-873 suppresses the translation of RIPK1/RIPK3 and inhibits RIPK1/RIPK3-mediated necroptotic cell death in cardiomyocytes. Hence, NRF contributes to necroptotic cell death in myocytes under oxidative stress. Furthermore, the detrimental role of NRF in I/R-induced cell death has also been verified in animal models of myocardial I/R injury, showing that intracoronary administration of NRF-siRNA prior to I/R

reduces the number of myosin-positive necrotic cells in the infarct region[39]. Also, thetumour suppressor p53 is involved in the induction of NRF expression in oxidant-treated myocytes[39].

In contrast to the anti-apoptotic role of H19 as discussed above, Wang et al.,using both in vitro and in vivo I/R models, have found thatH19 can inhibit necroptotic cell death by antagonizing miR-103/107 expression[40]. In myocytes, miR-103/107 facilitates necroptosis by targeting the signalling protein FADD, a negative regulator of the RIPK1/3 complex[40]. Indeed, a cytoprotective effect of H19 is also observed in cultured myocytes against hypoxia-induced injuries[41]. Therefore, whether H19 has a promoting or an inhibiting effect on myocardial cell death during I/R injury appears to be controversial.

### *3.4 LncRNAs Causing Autophagic Cell Death*

Autophagy maintains cellular homeostasis through the removal of dysfunctional proteins and organelles. However, excessive autophagy may potentially contribute to cardiac injury in both stages of ischaemia and reperfusion[42], although the nature of autophagic cell death in the heart tissue is still non-definitive[5].Recent evidence has demonstrated that lncRNA-mediated mechanisms are involved in modulating autophagy during myocardial I/R. For example, the lncRNA APF (autophagy promoting factor) has been found to act as an endogenous molecular sponge for miR-188-3p, which in turn negatively regulates the expression of ATG7, a keyfactor in mediating autophagy[43]. During myocardial I/R treatment, APF expression is significantly upregulated, leading to exaggerated autophagy and cell death[43]. As such, suppressing cardiac expression of APF with siRNA not only attenuates the expression of ATG7 and autophagy in I/R-treated hearts, but also reducesthe infarct size[43].

Kong and colleagues have shown that in H9c2 cardiomyocytes, overexpression of the lncRNA RMRP (component of mitochondrial RNA processing endoribonuclease) aggravates hypoxia-induced injury, likely via downregulating the expression of miR-206[44]. These authors confirmed that one target of miR-206 is ATG3, another key factor involved in mediating autophagy. Moreover, using an in vivo rat model of myocardial I/R injury, it has been demonstrated that suppression of RMPR improved cardiac functions and inhibited cell death. Paradoxically, the authors also identified a cytoprotective role of RMPR (via activation of the PI3K/Akt/mTOR pathway), while the precise role of increased autophagy in the detrimental effects of RMRP in vivo has not been firmly established either[44].

LncRNA AK139328 is found to be upregulated in the heart from diabetic models after myocardial I/R, while knockdown of AK139328 inhibits cardiomyocyte autophagy as well as apoptosis, and amelioratescardiac I/R injuries. In vitro, the authors have further confirmed that AK139328 facilitates I/R injury through modulating miR-204-3p, while miR-204-3p expression attenuates I/R injury via inhibiting cardiomyocyte autophagy[45].Likewise, Yin et al. haverevealed that Galont (GATA1 activated lncRNA), which is upregulated by I/R stimulus, can promote ATG5 expression and autophagic cell death in cardiomyocytes, via targetingmiR-338[46].

Beclin-1 is an important protein involved in mediating autophagy, and cells lacking beclin-1 lose the ability to form autophagic vacuoles[47]. Recently, Wang et al. have identified that the lncRNA AK088388 binds to

miR-30a and enhances the expression of beclin-1 and autophagy in I/R-treated cardiomyocytes, contributing to I/R-induced cellular damages[48].Interestingly, MALAT1, in addition to its pro-apoptotic effects in cardiomyocytes, has also been shown to promote autophagic cell death after I/R treatment[49]. In vitro, researchers have observed increased expressions of MALAT1 and beclin-1, and increased levels of autophagy in H9c2 cells treated with I/R, in parallel with cellular damages and cell death. Importantly, MALAT1 knockdown represses beclin-1-related cardiomyocyte autophagy by enhancing the expression of miR-20b-5p (of whichbeclin-1 is a molecular target), leading to improved cell viability[49].

### *3.5LncRNAsPreventingAutophagic Cell Death*

Some evidence suggests that lncRNAs can also inhibit autophagic cell death under pathological conditions. Liu et al. have characterized effects of the lncRNA CAIF (cardiac autophagy inhibitory factor), and shown that CAIF suppresses cardiac autophagy and attenuates myocardial injuries after I/R[50]. Unlike most of lncRNAs, the direct target of CAIF is not a microRNA, but a protein. Mechanistically, CAIF directly binds to p53 protein and blocks p53-mediated myocardin transcription, resulting in decreasedmyocardin expression[50]. Myocardin belongs to a family of transcription coactivators that regulate various cellular functions in cardiovascular cells[51]. Interestingly, it has been found that myocardin is upregulated in I/R-treated myocardium, and is involved in the induction of autophagic vacuole formation[50]. Consistent with this, knockdown of myocardin expression inhibits autophagy and attenuates myocardial infarction[50].

## 4. Molecular Mechanisms Underlying the actionsof LncRNAs in Myocardial I/R Injury

Among the principal molecular mechanisms of the lncRNA biology, available evidence has pointed to a pivotal role of ceRNA actions (or miRNA sponging) in mediating the effects of various lncRNAs in myocardial I/R injury (see Figure 2). A comprehensive summary of the ceRNA–miRNA–mRNA interaction networks involved in modulating myocyte injuries is given in a recent article by Xiong and colleagues[52]. Generally, a ceRNA antagonises the functions of one or more miRNAs, while miRNAs downregulate their target gene expressions by promoting mRNA degradation or hindering the process of translation. Therefore, an increased level of a lncRNA is anticipated to augment the protein expression of its target gene, unless in some special circumstances a miRNA enhances gene expression. Interestingly, it is noted that a single lncRNA in myocytes may affectmultiple targets which have similar biological effects (for example MALAT1 and GAS5, see Figure 2). In this case it is presumed that the final cellular phenotype is a summed result of the different pathways. On the other hand, some lncRNAs have been shown to interact with two miRNAs which have conflicting effects on the cell (for example H19, see Figure 2). It is still enigmatic how the counterbalancing activities of a lncRNA are coordinated in a single cell under stress conditions. Moreover, it appears that the functional effects and the underlying mechanisms of a single lncRNA, at least in the cardiovascular system, may be highly divergent in different tissues[8]. For instance, the lncRNA H19 may

interact with two different target miRNAs (namely miRNA-675 and miRNA-103) in myocytes (see Figure 2). In comparison, in aneurysmal vascular smooth muscles, H19 has been shown to enhance the binding of transcription factor Sp1 to the promotor of HIF-1$\alpha$ (hypoxia-inducible factor-1$\alpha$) gene, causing animpairment in proliferation and an increase in apoptosis in smooth muscle cells[53]. In the pulmonary vasculature, however, it has been reported that H19 interacts with the let-7 family of miRNAs, leading to increasedexpression of angiotensin II receptor type 1 and increasedsmooth muscle cell proliferation[54]. This pathway may contribute to the development of pulmonary arterial hypertension[54]. These findingstogether highlight a notion that the molecular mechanisms of lncRNA-mediated cardiovascular effects need to be addressed in a cell- and context-specific manner.

## 5. Are LncRNAs Potential Biomarkers for Ischaemic Heart Disease?

Patients with myocardial infarction show changes in circulating lncRNAs when compared to healthy controls[55,56]. Vausort et al. have characterized the expression of five cardiac-specific lncRNAs in peripheral blood cells isolated from patients with acute myocardial infarction, namely aHIF (hypoxia inducible factor 1A antisense RNA 2), ANRIL (cyclin-dependent kinase inhibitor 2B antisense RNA 1; also known as antisense non-coding RNA at the INK4 locus), MIAT (MI-associated transcript), KCNQ1OT1 (potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1), and MALAT1[55]. Levels of aHIF, KCNQ1OT1,and MALAT1are higher in patients with infarctionthan in healthy controls, while the level of ANRIL is lower.Of the five lncRNAs, ANRIL, KCNQ1OT1, MIAT, and MALAT1 maybe used as independent predictors for left ventricular dysfunctions in a 4-month follow-up period[55].Moreover, ANRIL expression level is associated with cardiovascular risk factors including age, diabetes mellitus, and hypertension[55]. Functionally, aHIF, ANRIL, MIAT and MALAT1 are considered to be related to the inflammatory response after myocardial infarction[55], and aberrant expression of ANRIL may beimplicated in vascular endothelial dysfunctions and coronary heart disease[57]. The serum level of ANRIL has also been shown to be a predictor ofprognosis in patients with coronary heart disease[58]. Likewise, the value of MALAT1 as a potential biomarker is also supported by another study showing that the expression level of MALAT1 is upregulated in the peripheral blood from patients with acute myocardial infarction[26].

Another biomarker candidate is the mitochondrial lncRNA LIPCAR.Global transcriptomic analyses have revealed that the plasma level of LIPCAR islowered early after myocardial infarction but upregulated during later stages[59]. LIPCAR predicts the development of cardiac remodellingafter infarction andappears to be an independent risk markerfor future cardiovascular deaths[59]. More recently, Li et al. have compared the circulating level of LIPCAR in patients with acute myocardial infarction before and after percutaneous coronary intervention[60]. It has been found that LIPCAR is significantly elevated in infarction patients as compared to the healthy controls, but the level is decreased after percutaneous coronary intervention. LIPCAR levels are positively correlated with myocardial enzymes (severity of infarction) and negatively correlated with left ventricular ejection fraction (cardiac functions). In addition, follow-up observations show that a higher level of LIPCAR is an independent predictor of major adverse cardiovascular events[60].

It is clear that levels of certain circulating lncRNAs show significant correlations to the clinical outcomeafter cardiac I/R injury. As the number of lncRNAs discovered are anticipated to rise rapidly, it is foreseen that multiple lncRNA molecules are to be identified as novel and reliable biomarkers for diagnosis or prognosis prediction ofischaemic heart disease. However, it is unclear whether these newly identified lncRNAs are better than the widely accepted protein biomarkers, since the majority of these clinical studies have not included a conventional biomarker like troponin for acute myocardial infarction[61]. Furthermore, thelimited yield of high-qualityRNA from the peripheral blood may restrict the clinical application of lncRNAs as novel biomarkers[62].

## 6.Concluding Remarks

Emerging *in vitro* and *in vivo* studies have identified an array of lncRNAs which are dysregulated in cardiomyocytes during I/R. Further functional studies have revealed that some of these lncRNAs are involved in determining the cellular outcome, including apoptosis, necroptosis and autophagic cell death, afterI/R injury (see Figure 2). These lncRNAs may have either cytoprotective or detrimental effects. It is noted that one potential limitation associated with lncRNA studies is the application of data obtained from animal models, sincethe majority of lncRNAsequences show poor species conservation (except for a few examples such as MALAT1)[63,64]. Nonetheless, mounting evidence has suggested that lncRNA functions are not solely determined by the primary nucleotide sequence, while other factors including secondary structures and spatio-temporal expression patterns are also important[63,64]. Hence, one lncRNA with low sequenceidentity between species may feasibly perform similar functions. It is proposed that targeting lncRNAsmay represent a new venue for combatingmyocardial I/R injury. Moreover, evidence has suggested that certain lncRNA molecules may be used as novel biomarkers for prognosis/diagnosis purposes in ischaemic heart disease.It is largely unknown, however, how I/R causes the dysregulation in lncRNA expressions, therefore further studies are warranted to delineate the mechanisms regulating lncRNA expressions.

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**Conflicts of interest**

The authors declare that they have no conflict of interest.

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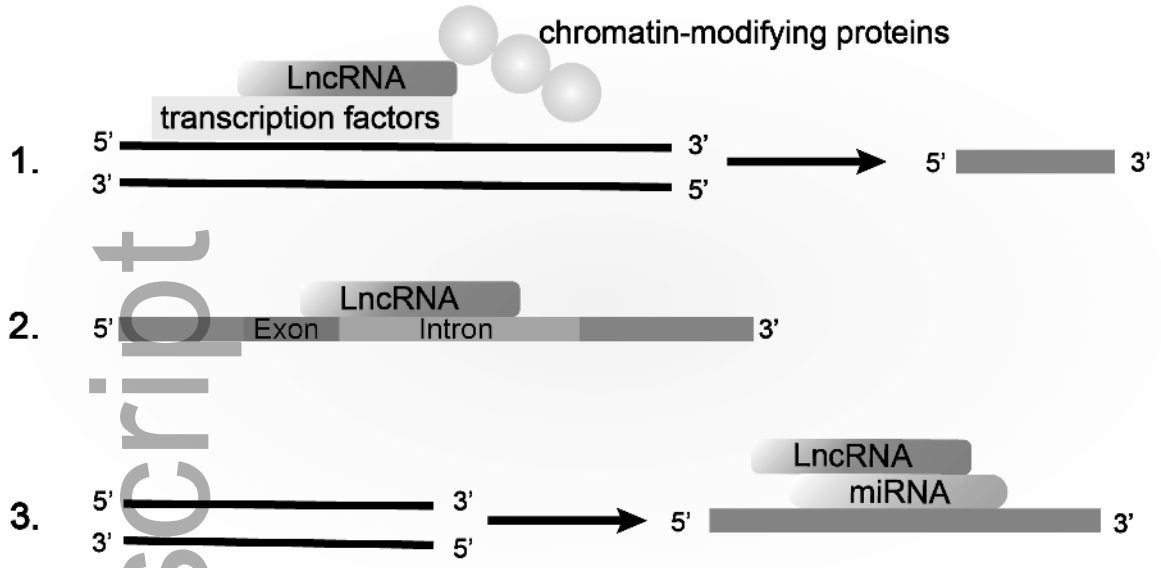
**Figure 1.** Major mechanisms by which lncRNAs regulate gene expression: (1) silencing or activating gene transcription by binding to transcription factors or chromatin-modifying proteins; (2) modulating mRNA stability or splicing; (3) indirectly affecting gene translation by interacting with miRNAs.

**Figure 2.** Illustration of some well documented lncRNAs (shown in light-blue boxes) involved in modulating cellular outcomes after myocardial I/R injury. Note that the cartoons do not necessarily represent direct physical interactions between lncRNAs and their targets; instead the regulation of intracellular targets by lncRNAs can be direct or indirect with varying mechanisms.

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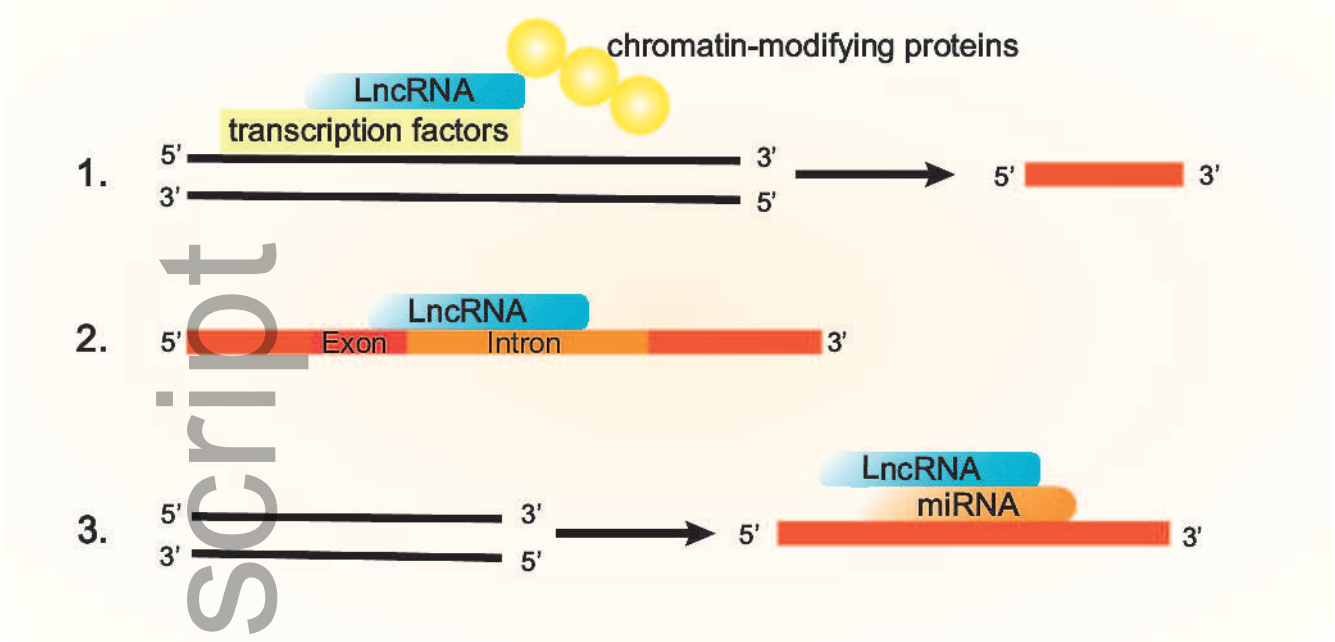


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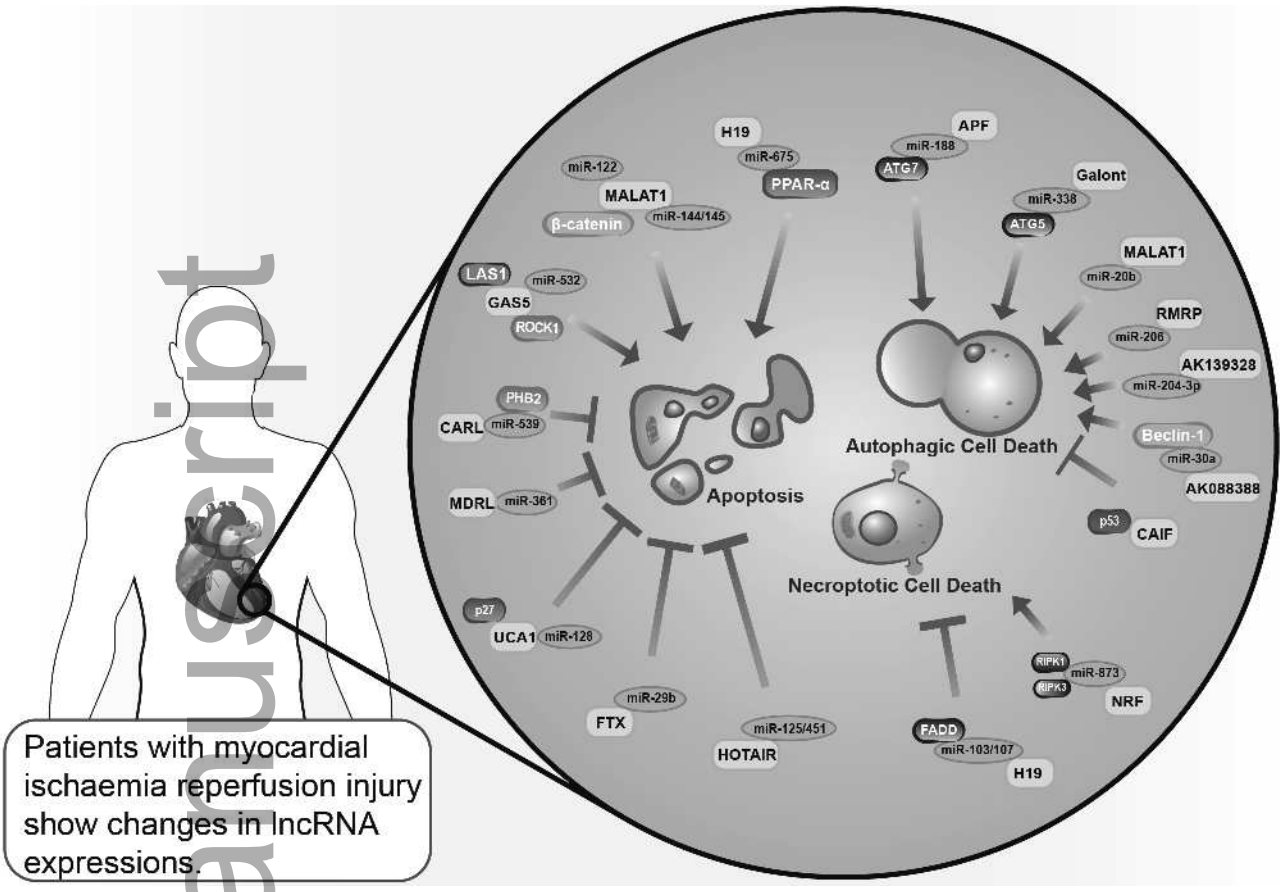


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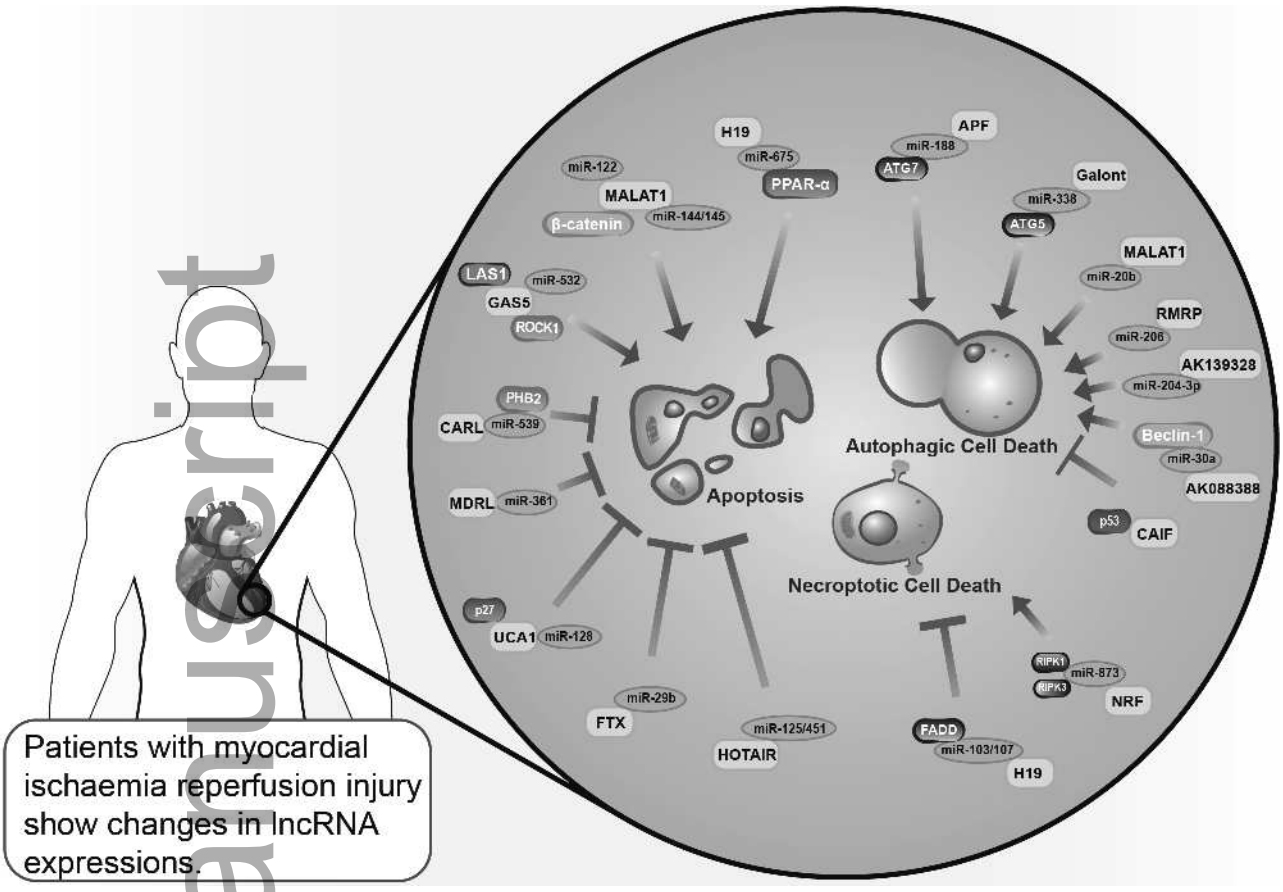


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