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Cardiac protection induced by urocortin-2 enables the regulation of apoptosis and fibrosis after ischemia and reperfusion involving miR-29a modulation

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Apoptosis

Fibrosis

CARDIAC
REMODELING

miR-29a
miR-103
miR-339-5
miR-423
miR-451_1*

I/R + Ucn-2

Transcriptional
regulation
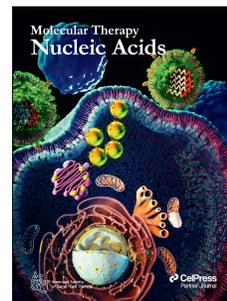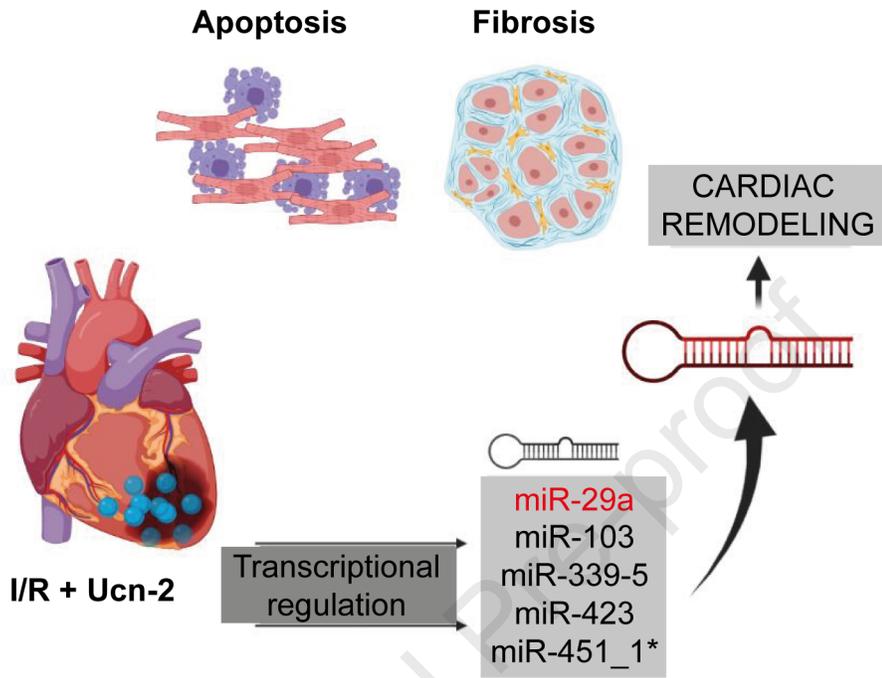
1 **Cardiac protection induced by urocortin-2 enables the regulation of apoptosis**

2 **and fibrosis after ischemia and reperfusion involving miR-29a modulation**

3

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36   **Short title:** Ucn-2 implicates miR-29a regulation in heart infarction

37 **<u>ABSTRACT</u>**

38 Urocortin-2 (Ucn-2) has demonstrated cardioprotective actions against

39 myocardial ischemia-reperfusion (I/R) injuries. Herein, we explored the

40 protective role of Ucn-2 through microRNAs (miRNAs) post-transcriptional

41 regulation of apoptotic and pro-fibrotic genes. We determined that the

42 intravenous administration of Ucn-2 before heart's reperfusion in Wistar rat

43 model of I/R recovered cardiac contractility and reduced fibrosis, LDH release,

44 and apoptosis. The infusion of Ucn-2 also inhibited the upregulation of 6

45 miRNAs in revascularized heart. The *in silico* analysis indicated that miR-29a and

46 miR-451_1* are predicted to target many apoptotic and fibrotic genes.

47 Accordingly, the transfection of neonatal rat ventricular myocytes with mimics

48 overexpressing miR-29a, but not miR-451_1*, prevented I/R-induced expression

49 of pro- and anti-apoptotic genes such as *Apaf-1*, *Hmox-1* and *Cycs*; as well as pro-

50 fibrotic genes *Col-I* and *Col-III*. We also confirmed that Hmox-1, target of miR-

51 29a, is highly expressed at mRNA and protein level in adult rat heart under I/R;

52 whereas, Ucn-2 abolished I/R induced mRNA and protein upregulation of

53 HMOX-1. Interestingly, a significant upregulation of *Hmox-1* was observed in the

54 ventricle of ischemic patients with heart failure, correlating negatively with the

55 left ventricle ejection fraction. Altogether, these data indicate that Ucn-2 through

56 miR-29a regulation, provides long-lasting cardioprotection, involving post-

57 transcriptional regulation of apoptotic and fibrotic genes.

58

59 **<u>Abbreviations:</u>**

60 AIFM-1, apoptosis-inducing factor 1, mitochondrial; AMI, acute myocardial

61 infarction; APAF-1, apoptosis protease-activating factor-1; BCL-2, B-Cell

62 lymphoma 2;; COL-I, collagen-I; COL-III, collagen-III; CYCS, cytochrome C; CRF-

63 R2, corticotropin releasing factor receptor 2; HF, heart failure; HMOX-1, heme

64 oxygenase 1; I/R, ischemia and reperfusion; LVEF, Left Ventricle Ejection

65 Fraction; MAPK-8, mitogen-activated protein kinase 8; miRNAs, microRNAs;

66 NVRMs, neonatal ventricle rat myocytes; pPCI, primary percutaneous coronary

67 intervention; TGF-β, transforming growth factor βeta; Ucn-2, Urocortin-2.

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76 **<u>INTRODUCTION</u>**

77   Acute myocardial infarction (AMI) is one of the major causes of morbidity

78 and mortality worldwide.[1] AMI sequelae, such as apoptosis of cardiac myocytes

79 in the so-called border or risk zone near the infarct scars are known to trigger the

80 adverse cardiac remodeling and aggravate cardiac dysfunction.[2,3] Benefits of

81 timely and effective early revascularization after AMI are well-recognized.

82 However, the process of myocardial revascularization is associated with critical

83 injuries that occur when oxygen rich blood re-enter the vulnerable myocardial

84 tissue, a phenomenon known as ischemia and reperfusion (I/R) syndrome.[4]

85 Lethal complications of I/R injuries cause the adverse cardiac remodeling and

86 consequently heart failure.[5,6] Therefore, effective strategies in cardioprotection

87 are still eagerly needed.

88   In recent years, evidences demonstrated that urocortin 2 (Ucn-2) has

89 cardioprotective effects on myocardial I/R injuries and heart failure.[7] Ucn-2 is an

90 endogenous peptide belonging to the corticotropin releasing factor (CRF) family.

91 Ucn-2 binds with high affinity to the receptor CRF-R2 that is highly expressed in

92 the cardiovascular system.[8] The administration of Ucn-2 evokes important

93 changes in the cardiovascular system, such as human coronary vasodilatation,[9]

94 and triggers potent cardioprotective effects against I/R injuries since it decreases

95 the infarct size and prevents harmful cell death.[10] Similarly, the other isoform

96 Ucn-1 induces positive inotropic and lusitropic effects in rats,[11] improves the

97 intracellular calcium concentration ($[Ca^{2+}]_i$) handling in I/R,[12] and efficiently

98   protects hearts from I/R injuries by the modulation of apoptotic genes, such as

99   *Cd40lg*, *Xiap* and *Bad*.[13] The infusion of Ucn-2 into the rat I/R-model also promotes

100   cardioprotection, involving changes in the expression of microRNAs (miRNAs),

101   which play major role in the post-transcriptional regulation of genes.[14] MiRNAs

102   are small non-coding RNAs that regulate a plethora of cellular processes related

103   to AMI, including cardiac myocyte apoptosis, necrosis and fibrosis.[15] They play

104   critical roles in heart function under pathophysiological conditions and also in

105   different cardioprotection strategies.[16,17] Recently, we demonstrated that the

106   levels of different miRNAs changed rapidly into the bloodstream of patients

107   suffering from AMI with ST-segment elevation (STEMI) undergoing primary

108   percutaneous coronary intervention (pPCI), and were related to the development

109   of the adverse cardiac remodeling.[18]

110   In the present study, we evaluated the role of Ucn-2 in the regulation of

111   miRNAs expression under I/R, focusing on a list of circulating miRNAs whose

112   levels changed in infarcted patients after pPCI. We further examined the role of

113   miRNAs in the regulation of pro-fibrotic and apoptotic genes induced by I/R.

114   **RESULTS**

115   **I/R increases circulating Ucn-2 and the expression of CRF-R2 in heart's tissue**

116   Since Ucn-2 is an endogenous stress-related peptide, we examined its

117   concentration in serum of I/R rat model following the experimental protocol

118   illustrated in Supplemental Figure 1A. Figure 1A shows that the concentration of

119   Ucn-2 increased significantly 1 week after heart's intervention, as compared to

120 sham, meanwhile it decreased 6 weeks after surgery. We also assessed the

121 expression of Ucn-2 receptor (CRF-R2) in risk zone of the infarcted heart. Figure

122 1B shows that the expression of CRF-R2 was significantly increased 1 week after

123 I/R, comparing to sham. In contrast, the expression of CRF-R2 was restored 6

124 weeks after surgery. Therefore, the level of circulating Ucn-2 and the expression

125 of CRF-R2 increased transiently after the heart infarction and its

126 revascularization.

127 **Ucn-2 recovers heart contractility and prevents I/R-induced fibrosis**

128   We investigated the cardioprotective effect of Ucn-2 (150 µg/Kg) infused 5

129 min before reperfusion, using different approaches. Data in Figure 2A and Table

130 1 indicate that 1 week after surgery the left ventricle ejection fraction (LVEF), the

131 fractional shortening (LVFS), as well as the left ventricular end-diastolic volume

132 (LVEdV) and the left ventricular end-systolic volume (LVEsV), recovered

133 significantly in Ucn-2 treated rats, as compared to I/R non-treated rats.

134 Meanwhile, I/R induced increase in the left ventricle diastolic diameter (LVdD)

135 was not affected by Ucn-2. Next, we examined the effect of Ucn-2 on cardiac

136 fibrosis. As shown in Figure 2B-D the administration of Ucn-2 decreased the

137 fibrotic areas of the infarcted hearts assessed *in vivo* by cardiac magnetic

138 resonance and by Masson's trichrome staining. Moreover, Figure 2E-H shows

139 that the expression of pro-fibrotic genes *Collagen*-I (*Col*-I), C*ollagen*-III (*Col*-III),

140 *Transforming Growth Factor β-1* (*Tgf-β1*) and *Transforming Growth Factor β-2* (*Tgf-*

141 *β2*) increased significantly in the risk zone of hearts isolated 1 week after I/R. By

7

142 contrast, rats treated with Ucn-2 showed significantly reduced expression of

143 these pro-fibrotic genes.

144 **Ucn-2 prevents I/R-induced apoptosis**

145 To further evaluate the cardioprotection exerted by Ucn-2, we examined its

146 action on cardiac myocytes viability and death. As illustrated in Figure 3A the

147 administration of Ucn-2 decreased significantly I/R-evoked lactate

148 dehydrogenase (LDH) concentrations, increased 24 h after I/R. Following the

149 experiment protocol outlined in Supplemental Figure 1A, Figure 3B shows that

150 Ucn-2 infusion in I/R rat markedly reduced the number of apoptotic cardiac

151 myocytes, as assessed by TUNEL assay. Ucn-2 treatment also trended to decrease

152 I/R-evoked caspase 3 cleavage (Figure 3C). To confirm these results, we examined

153 Ucn-2 effect on adult rat ventricle myocytes (ARVM), using annexin V staining.

154 As depicted in Figure 3D-F, Ucn-2 pre-treatment of cardiac myocytes exposed to

155 I/R decreased the number of apoptotic cells stained by annexin V, while it

156 preserved the number of living cells. Altogether, these data indicate that the

157 infusion of Ucn-2 at the onset of reperfusion preserves cardiac cell viability and

158 attenuates apoptosis.

159 **Ucn-2 modulates the expression of miRNAs in the heart under I/R**

160 Given the importance of the post-transcriptional regulation of the expression

161 of genes in cardiac pathophysiological processes,[14,19] we examined whether Ucn-

162 2 could regulate the expression of miRNAs in hearts excised from I/R rats model.

163 We selected a list of miRNAs based on the analysis of circulating miRNAs

164 released in patients with STEMI who underwent revascularization with primary

165 Percutaneous Coronary Intervention (pPCI), as described recently,[18] since little is

166 known about the role of those miRNAs in I/R and whether they can be good

167 target for cardioprotective drugs. Figure 4A shows a list of circulating miRNAs

168 released in serum of STEMI patients 3 h after the angioplasty. The expression of

169 miR-29a, miR-103, miR-125a-3p, miR-133, miR-139-3p, miR-320, miR-324-3p,

170 miR-324-5p, miR-339-5p, miR-423_1, miR-451_1* and miR-499-5p were also

171 detected in heart samples isolated from atrium biopsies of ischemic patients with

172 heart failure (HF) (Figure 4B). Based on these findings, we examined the

173 expression of these 12 human miRNAs in I/R rats' ventricle isolated from the risk

174 area. As illustrated in Figure 4C-H, the expression of miR-29a, miR-103, miR-133,

175 miR-339-5p, miR-423_1 and miR-451_1* was significantly upregulated at 24 h

176 and 1 week after I/R, except for miR-133 that showed a downregulation at 1 week

177 after I/R. In contrast, the administration of Ucn-2 5 min before heart's

178 revascularization prevented significantly I/R-induced upregulation of these

179 miRNAs, excluding miR-423_1 which was not sensitive to Ucn-2 24 h after I/R.

180 Supplemental Figure 2 shows that I/R evoked significant increase in the

181 expression of miR-125a, miR-139, miR-320, and miR-324-3p at 24 h, but not of

182 miR-324-5p and miR-499_1, while the administration of Ucn-2 did not prevent

183 the overexpression of these miRNAs. Therefore, Ucn-2 efficiently modulated the

184 expression of some miRNAs associated with pPCI in STEMI patients, which are

185 altered by I/R in heart tissue of rats.

186   **miR-29a and miR-451_1* are predicted to modulate the expression of genes**

187   **related to apoptosis and cell survival pathway**

188   To determine target genes of miR-29a, miR-103, miR-133, miR-339-5p, miR-

189   423_1 and miR-451_1*, we performed an *in silico* analysis using PANTHER

190   software. As illustrated in Figure 5A the analysis generated a pie chart suggesting

191   that miRNAs have predicted target genes are involved in pathways of apoptosis

192   and fibrosis. Specifically, 41 signaling pathways are mainly implicated in cellular

193   processes associated with post-AMI, such as apoptosis and fibrosis. Interestingly,

194   we found that only miR-29a and miR-451_1* are predicted to target 16 and 17

195   apoptotic genes, and 14 and 18 genes related to fibrosis, respectively (Figure 5B).

196   Based on this analysis and to assess the role of miR-29a and miR-451_1* in the

197   regulation of those predicted genes we performed the quantitative RT PrimePCR

198   array, using the Bio-Rad predesigned assay specifically for apoptosis and

199   survival pathway (Supplemental Figure 3A). Experiments were performed in

200   neonatal rat ventricular myocytes (NRVM) transfected with mimics of miRNAs

201   to overexpress miR-29a and miR-451_1*, under *in vitro* I/R protocol as explained

202   in Supplemental Figure 1B. First, we checked if the expression of miR-29a and

203   miR-451_1* are similarly sensitive to I/R in NRVM and adult heart. Accordantly,

204   Supplemental Figure 3B and C confirms that I/R enhanced the expression of miR-

205   29a and miR-451_1*, while Ucn-2 significantly inhibited both miRNAs in a

206   similar way in NRVM and in adult rat heart. Second, Supplemental Figure 3D

207   and E shows that NRVM transfection with mimics of miR-29a and miR-451_1*

208   successfully increased the levels of miR-29a and miR-451-1*.

209   Figure 5C and Supplemental table 1 show that 56 genes were upregulated and

210   20 downregulated 24 h after I/R in NRVM. By contrast, in NRVM transfected with

211   mimics of miR-29a and miR-451_1*, 56 and 49 genes were downregulated, while

212   20 and 27 genes were upregulated, respectively (Figure 5D and 5E; Supplemental

213   table 1). These data indicate that mimics of miR-29a and miR-451_1* reverted the

214   expression of many apoptotic genes overexpressed under I/R.

215   **miR-29a regulates the expression of I/R-induced apoptotic and fibrotic genes**

216   To verify the results of the PrimePCR array we examined the expression of 6

217   selected genes in NRVM transfected with mimics of miR-29a and miR-451_1*

218   under I/R. The selection of these genes was based on their fold change rates as

219   well as their implication in I/R-related processes, as published elsewhere.[20–22]

220   Namely, we investigated the expression of *Apoptosis Inducing Factor mitochondria*

221   *associated 1* (*Aifm1*), *Apoptosis Protease-Activating Factor-1* (*Apaf1*), *B-Cell Lymphoma*

222   *2* (*Bcl-2*), *Cytochrome C* (*Cycs*), *Heme Oxygenase 1* (*Hmox-1*) and *Mitogen-Activated*

223   *Protein Kinase 8* (*Mapk-8*). Figure 6A and B shows that the expression of *Apaf-1*

224   and *Hmox-1* increased significantly in I/R, as compared to control. Figure 6C

225   indicates that the expression of *Cycs* slightly increased under I/R, although not

226   significantly. However, the expression of *Aifm-1*, *Bcl-2* and *Mapk-8* was not

227   affected by I/R, as compared to control (Figure 6D, E and F). Conversely, mimic

228   of miR-29a, but not of miR-451_1*, prevented I/R effects on *Apaf-1*, *Hmox-1*, and

229   *Cycs* (Figure 6A-C). Meanwhile, miR-29a enhanced the expression of *Aifm-1*

230   (Figure 6D), and decreased the expression of *Mapk-8* (Figure 6F) under I/R. In

231   contrast, miR-451_1* mimic significantly increased the expression of *Hmox-1* and

232   *Mapk-8,* comparing to their levels in I/R (Figure 6C and F). The expression of *Bcl-*

233   *2* was not affected either by I/R or miRNAs mimics. Moreover, we analyzed if

234   miR-29a and miR-451_1* could target pro-fibrotic genes in NRVM, as is the case

235   of the effect of Ucn-2 in tissue of adult heart shown previously in Figure 2. Figure

236   6G and H shows that I/R induced small but significant upregulation of *Col-I* and

237   *Col-III*, which was significantly downregulated by miR-29a. Of note, miR-451_1*

238   also failed to modulate the expression of *Col-I* and *Col-III*. These data indicate that

239   miR-29a, but not miR-451-1*, modulated I/R-induced overexpression of *Apaf-1*,

240   *Hmox-1*, *Cycs*, *Col-I* and *Col-III*.

241   **<u>Signaling pathway involved in miR-29a regulation by Ucn-2</u>**

242   Once we determined that miR-29a efficiently modulated I/R-induced changes

243   in the expression of apoptotic and fibrotic genes, we studied the signaling

244   pathway involved in the regulation of miR-29a by Ucn-2 applied in NRVM under

245   I/R (Supplemental Figure 1C). Figure 6I shows that NRVM treatment with Ucn-

246   2 (10 nM) before reperfusion inhibited I/R-induced miR-29a overexpression,

247   whereas NRVM pretreatment with astressin (0.5 μM), the specific antagonist of

248   CRF-R2 receptor,[23] significantly attenuated Ucn-2 effect. CRF-R2 is known to

249   couple Gs/cAMP/PKA signalling, therefore we investigated if Ucn-2 action was

250   mediated by of PKA or Epac (exchange protein directly activated by cAMP). Our

251 data show that NRVM pretreatment with ESI-05 (10 μM), specific inhibitor of

252 Epac2,[24] abolished Ucn-2 downregulation of miR-29a. By contrast, PKA

253 inhibition with H89 (1 μM) did not significantly affect Ucn-2 downregulation of

254 miR-29a. Finally, because Epac activates the Ras1-ERK1/2 pathway,[26] we

255 examined whether ERK1/2 participates in Ucn-2 action. Nevertheless, the

256 inhibition of ERK1/2 by PD 098059 (5 μM)[28] did not inhibit significantly Ucn-2-

257 effect on miR-29a under I/R. Altogether, these data demonstrate that the

258 administration of Ucn-2 before reperfusion modulated the expression miR-29a

259 through the activation of CRF-R2 and Epac2.

260 **<u>I/R induced changes in the expression of apoptotic genes in rat's ventricle</u>**

261   To confirm if these genes are relevant in the adult infarcted heart, we

262 examined their expression in I/R rats infused with Ucn-2. Figure 7A-C shows that

263 mRNA expression of *Hmox-1*, *Aifm-1* and *Apaf-1* were increased in risk zones 24

264 h after I/R, but it significantly decreased 1 week after the intervention. Moreover,

265 the administration of Ucn-2 blocked I/R-evoked *Hmox-1* upregulation, while it

266 enhanced I/R-induced expression of *Aifm-1*. In contrast, Figure 7D shows that

267 mRNA expression of *Cycs* significantly decreased 24 h, but it recovered after 1

268 week after I/R. The expression of *Cycs* as well as *Apaf-1* was not affected by Ucn-

269 2 (Figure 7C, D). At the same time, we did not observe significant changes in the

270 expression of *Mapk-8* under any experimental conditions, while Ucn-2 induced

271 *Bcl-2* increase 24 h after I/R (Supplemental Figure 4). Interestingly, as shown in

272 Figure 7E and F the protein expression of HMOX-1 and AIFM-1 were

13

273 significantly increased in risk zone of I/R rats 24 h after surgery, but Ucn-2

274 potently reduced HMOX-1 expression and tended to decrease the upregulation

275 of AIFM-1. By contrast, CYCS protein was not affected by I/R nor by Ucn-2

276 (Figure 7G).

277 Finally, we assessed the expression of these genes in ventricle biopsies of

278 patients with HF of ischemic origin. Figure 8A shows that only the expression of

279 *Hmox-1* was significantly increased, as compared to a healthy ventricle sample.

280 The expression of *Apaf-1* and *Aifm-1* slightly but not significantly trend to

281 increase in those patients. Meanwhile, the expression of *Mapk-8* and *Cycs* tended

282 to decrease in these samples. Interestingly, the analysis of a possible correlation

283 between patients' LVEF and these genes expression shows significant negative

284 correlations between the expression of *Hmox-1*, *Mapk-8*, and *Cycs* with the LVEF

285 of the patients, whereas the expression of *Apaf-1* and *Aifm-1* did not correlate with

286 the LVEF (Figure 8B-F). Altogether, these results suggest that HF patients might

287 overexpress genes related to apoptosis in function of the severity of their HF,

288 although higher number of samples is necessary to confirm this preliminary

289 observation.

290 **DISCUSSION**

291 Despite the overwhelming advances in cardiovascular therapies HF

292 following AMI remains the leading cause of mortality and morbidity in humans.

293 Therefore, strategies of cardioprotection are of major interest to limit I/R injuries

294 and cardiac myocytes loss after AMI.[25] This study confirms the important

295 protective role of the administration of Ucn-2 at early reperfusion which

296 mitigates I/R injuries. We observed significant and transient increase in

297 circulating Ucn-2 and the expression of its receptor CRF-R2, 1 week after I/R. This

298 result agree with previous studies which proposed Ucn-2 as a potential

299 diagnostic and prognostic biomarker for cardiovascular diseases.[27,29] Ucn-2

300 belongs to the stress hormone CRF family; therefore, our data confirm that under

301 the stress caused by I/R, the heart enhances not only the circulating Ucn-2 but

302 also its CRF-R2 receptor to activate the related signaling pathway in the injured

303 heart. Moreover, we demonstrate that intravenous infusion of Ucn-2 improves

304 cardiac contractility after I/R since it increases LVEsV and decreases the LVEdV,

305 indicating successful heart contraction and relaxation. In addition, using

306 different approaches we demonstrate that Ucn-2 decreases significantly I/R-

307 induced fibrosis, which will preserve myocardial compliance and will prevent

308 impaired cardiac diastolic and systolic function evoked by I/R.

309   I/R-induced cardiac cells death in affected hearts is another important factor

310 contributing to cardiac dysfunction and cardiac remodelling. Here, we

311 demonstrate that Ucn-2 reduces LDH amount, and we observe less cleaved

312 caspase 3 staining and DNA fragmentation, indicative of apoptosis, in Ucn-2

313 infused I/R rats. Annexin V staining further confirms that Ucn-2 prevents ARVM

314 death and increase cell survival, in accordance with our previously published

315 data.[10,14]

316   One of the limitations of the infusion of cardioprotective drugs is related to

317   their limited benefits duration due to their short half-life. Recent studies

318   indicated that Ucn-2 gene transfer provides sustained increase in the

319   concentration of plasma Ucn-2 and enhanced cardiac function in normal mice

320   and in mice with HF,[30,31] although its role in modulating I/R stress was not

321   assessed. Herein, we provide evidences demonstrating that Ucn-2 modulates

322   changes in miRNAs, post-transcriptional gene expression, and protein

323   expression, in agreement with previous studies.[14,32] We decided to study the

324   effect of Ucn-2 on miRNAs which have been recently detected in blood samples

325   of STEMI patients undergoing pPCI,[18] and in failing heart samples, because little

326   is known about the role of those human miRNAs in cardiac function after I/R.

327   Our results using adult rats and isolated cardiac myocytes unveil the ability of

328   Ucn-2 to modulate the expression of 6 of those miRNAs that are rapidly released

329   to the blood stream after pPCI in STEMI patients. In fact, we demonstrate that

330   Ucn-2 infusion prevents I/R-evoked upregulation of miR-29a, miR-103, miR-133,

331   miR-339-5p, miR-423_1 and miR-451_1, in rats. This effect was even sustained 1

332   week after heart reperfusion, indicating at least a medium lasting action of Ucn-

333   2 on miRNAs dysregulation.

334   Based on the *in silico* and PrimePCR findings, we found that miR-29a and

335   miR-451_1* possibly target many genes associated with apoptosis and fibrosis,

336   two prevalent pathways during the early adverse cardiac remodeling. We

337   demonstrate that the overexpression of miR-29a, but not miR-451_1*, efficiently

338   prevent the expression of collagen mRNA, indicating fibrosis inhibition in

339   agreement with recent studies which showed that miR-29a inhibits fibrosis in

340   myocardial infarcted rats,[33] in heart stressed with isoproterenol by

341   downregulating the expression of *DNA Methyltransferase enzymes A* (*Dnmt3a*),[34]

342   and in heart derived from chemotherapy.[35] Furthermore, miR-29a overexpression

343   prevents I/R-induced upregulation of *Apaf-1*, *Cycs*, and *Hmox-1*; meanwhile it

344   increases the expression of *Aifm-1*. As known, the intrinsic mitochondrial

345   apoptotic pathway is initiated after reperfusion by the release of *Cycs* into the

346   cytoplasm which stimulates *Apaf-1* and procaspase-9 in the apoptosome,

347   inducing apoptosis.[36–38] Thus, *Apaf-1*, *Aifm-1* and *Cycs* are considered pro-

348   apoptotic genes. By contrast, *Hmox-1* is considered anti-apoptotic and

349   cardioprotective. For instance, its gene delivery prevents cardiac remodeling and

350   preserves cardiac function after myocardial infarction, as described previously.[39]

351   Other studies demonstrated that the transplantation of mesenchymal stem cells

352   overexpressing *Hmox-1* conferred cardioprotection against ischemic injury in

353   heart and skeletal muscle.[40,41] There is a general consensus that cardiac myocyte

354   activates both pro- and anti-apoptotic pathways during the progressive

355   transition of the heart from a situation of adaptation to one of maladjustment

356   after I/R.[42] Therefore, miR-29a and its predicted target genes could be a potential

357   regulator of a balance between pro- and anti-apoptotic processes. miR-29a has

358   been reported to play other beneficial role in cardiovascular homeostasis, such as

359   cardiac hypertrophy,[43] and modulation of cardiac cell metabolism,[44] indicating

360   their potential features as therapeutic agent.

361   In this study, we also show in adult rat heart that HMOX-1 can be regulated

362   by Ucn-2, in the same way as miR-29a, both at mRNA and protein levels. By

363   contrast, Ucn-2 modulates differentially AIFM-1 at mRNA and protein levels,

364   indicating that perhaps Ucn-2 affects the post-translational process of some

365   protein. This finding suggest that the protective effect of Ucn-2 does not occur

366   exclusively through miR-29a and may involve other mediators that could act

367   differentially in post-transcriptional and post-translational processes.

368   Interestingly, we demonstrate that Ucn-2 regulates the expression of miR-29a

369   through the activation of CRF-R2 and Epac2, which is consistent with the role of

370   Epac2 on miR-139-3p and miR-324 modulation by Ucn-1 isoform in cardiac

371   myocytes.[14]

372   Furthermore, we provide preliminary data showing that ischemic patients

373   with HF overexpress *Hmox-1*, while the expression of other apoptotic genes seem

374   not significantly altered. We demonstrate a negative correlation between LVEF

375   of HF patient and the expression of *Hmox-1*, *Cycs* and *Mapk-8*, which may be

376   related to the *Cycs*-mediated cell death pathway. Although, the overexpression

377   of *Hmox-1* was unexpected since this gene is sought to exert anti-inflammatory

378   and anti-apoptotic effects post-AMI.[45] Perhaps, the overexpression of *Hmox-1*

379   may play a role in sustaining and protecting the still non-affected tissue of the

380 infarcted heart in those HF patients when their LVEF is severely compromised.

381 Further experiments are needed to clarify these data.

382   To summarize, this study demonstrate that Ucn-2 provides long-lasting

383 cardioprotective effects involving miRNAs regulation, which target apoptosis

384 and fibrosis. Mimicking changes of the expression of miRNAs caused by Ucn-2,

385 combined with functional studies, allows us to efficiently identify new role of

386 miR-29a in myocardial I/R, that presumably leads to a balanced regulation of

387 anti- and pro-apoptotic pathways.

388

389 **<u>MATERIALS AND METHODS</u>**

390 This study was performed in accordance with the recommendations of the Royal

391 Decree 53/2013 in agreement to the Directive 2010/63/EU of the European

392 Parliament and approved by the local Ethics Committee on Human and animal

393 Research of A Coruña and University Hospital of Virgen del Rocio of Seville.

394 **<u>Blood human samples and myocardial biopsies from patients with heart</u>**

395 **<u>failure</u>**

396 Human serum was obtained from blood samples from patients who suffered a

397 first STEMI by centrifugation at 1500 g for 15 min, as detailed in previously.[18] The

398 inclusion criteria were patients under 75 years old, diagnosed with AMI,

399 presenting symptoms 2 to 6 h prior to angioplasty and exhibiting epicardial TIMI

400 (Thrombolysis in Myocardial Infarction) flow grade of 0 in the initial angiogram.

401 Patients with a previous history of ischemic heart disease, a glomerular filtration

402 rate less than 30 mL/min, TIMI flow grade > 1 at the time of angiography were

403 excluded. Patients received standard pharmacological therapy as per current

404 clinical guidelines.

405 Myocardial biopsies were obtained from the atrium of 5 males and 2 females with

406 a median age of 62 years, and LVEF = 52.8 ± 2.1%, before surgery. Left ventricle

407 biopsies of ischemic patients with HF were obtained from 4 males and 3 females,

408 with a median age of 58.6 years and LVEF = 31.7 ± 6.4%. We also used one

409 ventricle biopsy from a healthy donor. These samples were obtained from

410 patients during surgery for cardiac transplantation at the University Hospital of

411 Virgen del Rocio in Seville and in A Coruña Hospital. A signed informed written

412 consent was provided from the families of all donors.

413 **Rat model of myocardial Ischemia and Reperfusion (I/R)**

414 The I/R rat model was performed using male Wistar rats weighing 250 ± 50 g as

415 previously described.[10] Briefly, rats were anesthetized with intraperitoneal (i.p.)

416 injection of 50 mg/Kg ketamine plus 8 mg/Kg xylazine and were maintained with

417 a mixture of 2% O_2/sevoflurane during the whole procedure. A left thoracotomy

418 was performed in the intercostal space followed by a pericardiotomy. To induce

419 the stenosis a 6/0 Prolene™ (Ethicon™, NJ, US) nylon suture was placed around

420 the left anterior descendent coronary artery, reducing the vascular light using a

421 small piece of PE-10 tube that was placed-in-between for a convenient release

422 upon reperfusion. Analgesia was provided during the 3 days following surgery.

423 ***In vivo* experimental groups**

424 As shown in Supplemental Figure 1A, experimental group in rat model were

425 divided in: "Sham" group: rats undergoing the same surgical procedure without

426 coronary ligation. "I/R" group: ischemia was produced by ligation of the left

427 coronary artery during 40 min, afterward 0.9% NaCl solution was added through

428 tail veins 5 min before reperfusion. "I/R + Ucn-2" group: Same as I/R group but

429 i.v. dose of Ucn-2 (150 μg/Kg) was administered 5 min before reperfusion.

430 Experiments were performed especially in risk zone of the infarcted heart, which

431 belongs to the adjacent areas of the artery ligature.[10,46] Three end points (24 h, 1

432 week and 6 weeks) were used depending on the experiment.

433 **Adult rat ventricle myocytes (ARVMs) primary culture**

434 The hearts were removed and mounted on a Langendorff perfusion apparatus.

435 Adult rat ventricle myocytes (ARVM) were isolated using collagenase type II (251

436 IU/mL) (Worthington Biochemical, CA, US) as described previously R.[12] Isolated

437 cells were filtered, centrifuged, and suspended in Tyrode solution containing

438 (mM): 130 NaCl, 1 CaCl2, 0.5 MgCl2, 5.4 KCl, 22 glucose, 25 HEPES, 0.4

439 NaH2PO4, 5 NaHCO3) (pH 7.4). ARVM were plated in control solution

440 containing 1.8 mM CaCl2 at 37°C and were later subjected to a protocol of I/R as

441 summarized in Supplemental Figure 1B, using a simulated ischemic solution

442 (mM): 142 NaCl, 3.6 KCl, 1.2 MgCl2, 1.8 CaCl2, 5 NaHCO3, 20 Hepes, 20 Lactate-

443 Na, 20 sucrose (pH 6.22), as described previously.[14,47] Cells were placed during 30

444 min in an incubator at 1% O2 and 5% CO2. Afterward, cells were reoxygenated

445 in control solution and maintained in at 21% O2 and 5% CO2 for 18-24 h. 30 nM

446   Ucn-2 was added before reperfusion. All experiments were performed on Ca^{2+}-

447   tolerant rod-shaped myocytes.

448   **Neonatal Rat Ventricle Myocytes (NRVMs) primary culture**

449   Neonatal Rat Ventricle Cardiac myocytes (NRVMs) were isolated from the heart

450   of 1-3 days old Wistar rats. The primary ventricular cardiac myocytes were

451   cultured in Dulbecco's Modified Eagle Medium (DMEM)/medium 199 (4:1)

452   supplemented with 10% horse serum, 15% fetal bovine serum (FBS), 1%

453   glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin for 24 h, later the

454   medium was replaced.

455   For miRNAs experiments, NRVMs were transfected at 70% of confluence, 48 h

456   after isolation, according to the manufacturer's instructions, using Lipofectamine

457   RNAiMAX Transfection Reagent (Thermo Fisher Scientific, US) with 10 μM of

458   mimics Rno -miR 29a (5′ UAGCACCAUCUGAAAUCGGUUA 3′), Rno -miR 451

459   (5′AAACCGUUACCAUUACUGAGUU 3′) or negative-control (Ambion,

460   Thermo Fisher, MA, US). 24 h later cells were exposed to the protocol of I/R as

461   illustrated in the Supplemental Figure 1B.

462   For pharmacological study, NRVM were incubated with inhibitors 10 min before

463   their exposition to I/R ± Ucn-2 (Supplemental Figure 1C).

464   **Echocardiography and Cardiovascular Magnetic Resonance**

465   Transthoracic echocardiographic and the cardiac magnetic resonance analysis

466   were performed as described previously[10]. The cardiac function was assessed 1

467   week after surgery in light anesthetized rats with 2% sevoflurane by Vevo™ 2100

468　ultrasound system with a transducer MS250 using a frequency range of 13-24

469　MHz (VisualSonics™, Toronto, Canada). M-Mode images of the left ventricle at

470　the level of the papillary muscles were obtained, and functional hemodynamic

471　parameters were recorded as the left ventricle ejection fraction (LVEF), left

472　ventricle ejection fraction; (LVFS), left ventricle end diastolic diameter (LVEdD),

473　left ventricle end-diastolic Volume (LVEdV), and left ventricle end-systolic

474　Volume (LVEsV).

475　The cardiac magnetic resonance study was performed with the imaging system

476　ICON 1T (Bruker, Rheinstetten, Germany) using a rat whole body coil. To

477　quantify the ischemic area, images were collected with gradient echo T1

478　sequences and synchronized with the electrocardiogram (repetition time: 100 ms,

479　echo time: 2.5 ms, resolution: 0.234 × 0.234 mm, slice thickness: 1.250 mm, angle

480　of rotation: 75° or 90°, 2 cuts with the same geometry as the previous film

481　sequences, 15 min after the introduction of a gadolinium-based contrast to

482　highlight fibrotic areas. The acquisition of images and their analysis were

483　performed in a blind manner.

484　**Masson´s trichrome staining**

485　Hearts from the three experimental groups were fixed with formalin and

486　embedded in paraffin. Hearts were cut into 6 μm sections and Masson's

487　trichrome protocol was performed to determine fibrosis stained in blue. Tissue

488　without fibrosis was stained in red.

489　**Tunnel assay and cleaved caspase 3 immunofluorescence.**

490   Hearts of rats were dissected, washed in cold PBS for blood clearance, and fixed

491   with 4% paraphormaldehyde. Sections were immersed in OCT and frozen to -

492   80ºC and later cut in 6 μM slices. For Tunnel assay, heart sections were stained

493   using the in-situ Cell Death Detection Kit, with fluorescein (Roche, Basel,

494   Switzerland) following the instruction of the manufacturer. Tunnel-positive

495   nuclei fluoresced bright green at 480–500 nm. Images were taken by widefield

496   Thunder microscope Leica (Leica, Westzlar, Germany) with computational

497   clearance at 10X.

498   For Caspase 3 immunofluorescence, heart sections were incubated overnight

499   with primary anti-cleaved Caspase 3 antibody (Cell Signalling, MA, US). After

500   washing, sections were incubated with a secondary antibody Alexa Fluor 594

501   (Thermo Fisher Scientific, MA, US). Wheat germ agglutinin (WGA) conjugated

502   with Alexa-fluor 488 was used to stain cell membrane, while DAPI was used to

503   stain nuclei. Five snapshots per condition were acquired using a fluorescence

504   microscope Olympus BX61 (Tokio, Japan) with 40X objective, and images were

505   analyzed to count the different proportion of red stained cells with ImageJ 1.45

506   software (Wayne Rasband, National Institute of Health, Bethesda, MD, USA).

507   **<u>Annexin V-FITC staining</u>**

508   Annexin V-FITC staining was used to detect apoptosis in adult rat cardiac

509   myocytes seeded in 6-channel μ-Slides VI 0,4 (Ibidi, Gräfelfing, Germany),

510   incubated with 100 μl of the binding buffer supplied with the kit (Trevigen, MN,

511   US), and 1 μl of annexin-FITC reagent, during 35 min at 25°C. Images were taken

512 with confocal microscope Leica TCS SP2 (Leica, Westzlar, Germany). Five

513 snapshots per condition were acquired using a HCX PI Apo CS dry 20X objective

514 with 2x zoom in z-stacks intervals, and maximum projection was recorded and

515 analyzed with Image J software ImageJ 1.45 software (Wayne Rasband, National

516 Institute of Health, Bethesda, MD USA), to count the proportion of labeled cells.

517 All assays were performed per triplicate, and counts were independently

518 conducted by two people in a blind manner.

519 **Elisa**

520 Serum of rats from the 3 experimental groups were purified using separation

521 columns. The level of Ucn-2 was determined by immunofluorescence assay

522 (Phoenix Pharmaceuticals, CA, US) following manufacturer instructions. The

523 level of LDH was detected by LDH-Glo™ Cytotoxicity Assay (Promega, WI, US).

524 **RNA extraction and qRT-PCR analysis**

525 miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany) was used to extract

526 small RNAs from patient´s serum following the manufacturer instructions.

527 Taqman® array miRNAs cards pool A (Applied Biosystem, CA, US) was also

528 used to determine changes in the expression of miRNA using Viia7 Real-Time

529 PCR system (Applied Biosystems, CA, US). The plate design includes

530 mammalian U6, RNU44 and RNU46 as endogenous control.

531 For tissue samples, miRNeasy kit (Qiagen, Hilden, Germany) was used to extract

532 total RNA or miRNAs following manufacturer instructions. Samples were

533 quantified using Nanodrop (Thermo Fisher Scientific, MA, US) for RNA and by

534   Qubit™ (Thermo Fisher Scientific, MA, US) for miRNAs. Reverse transcription

535   reactions were performed using miScript II RT Kit (Qiagen, Germany) (500 ng),

536   in accordance with the manufacturer's protocols. Prior to qRT-PCR reactions,

537   cDNA was diluted 1 in 5 for PCR assays.

538   PCR assays of miRNAs were performed using 10X universal primer (miScript

539   SYBR Green PCR Kit, Qiagen, Hilden, Germany), Sybr Green reactive (iTaq™

540   Universal SYBR Green Supermix, Bio-Rad, CA, US), and specific oligos of each

541   miRNA: miR-103, miR-125a, miR-133, miR-139, miR-29a, miR-320, miR-324-3p,

542   miR-324-5p, miR-339-5p, miR-423_1, miR-451_1* and miR-499_1 (Qiagen,

543   Hilden, Germany) according to the manufacturer instructions, using a ViiA 7

544   Real-Time PCR System (Applied Biosystems, MA, US). The average expression

545   levels of miRNAs in cells were normalized to miRTC1.

546   Reactions for genes PCR assays were performed in a 10 µL reaction mixture

547   volume with 100 nM forward primer and 100 nM reverse primer for mRNA.

548   Primer sequences are described in Supplemental Table 2. The average expression

549   levels of genes were normalized to β-actin.

550   ViiA 7 Software version 1.2 (Life Technologies, Carlsbad, CA, USA) was used to

551   calculate the quantification cycle (Ct) value, which is defined as the number of

552   cycles at which the fluorescence signal is significantly above the threshold;

553   expression of each mRNA and miRNA was defined from the threshold cycle (Ct),

554   and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after

555   normalization with the internal control, miRTC1 and β-actin for miRNA and

556   mRNA, respectively. Data are expressed as relative expression of Log Fold

557   change of means ± Standard Error of the Mean (± SEM) of at least 4 replicates of

558   each experiment.

559   **PrimePCR assay**

560   We used PrimePCR (Bio-Rad, CA, US) predesigned assay (Apoptosis and

561   Survival Tier 1 H96), containing primers of validated genes specifically for

562   apoptosis and survival pathway, as indicated in Supplemental table 1 and figure

563   2. A mix of 4 samples were added and the reaction was performed according to

564   the manufacturer's instructions, on a Viia7 Real-Time PCR system (Applied

565   Biosystems, CA, US). The average expression levels of genes were normalized to

566   the expression of housekeeping gene HPRT1.

567   ***In silico* analysis of targeted genes by miRNAs**

568   The analysis of targeted genes predicted to be regulated by miRNAs was done

569   using different bioinformatics resources: miRBD (miRDB v7.2, http://mirdb.org,

570   Washington University St. Louis, MO, US) ,TargetScan (Release 7.1,

571   www.targetscan.org, Cambridge, MA, US) databases, and Exiqon tool

572   application (http://www.exiqon.com/microrna-target-prediction). To identify

573   miRNA target gene pathways, we also used the online platform Gene Ontology

574   (GO) browser PANTHER (Protein Analysis THrough Evolutionary

575   Relationships) v14.1, (http://pantherdb.org/genelistanalysis.do, University of

576   Southern California, Los Angeles, CA, US).

577   **Western Blotting**

578   Protein extraction was carried out using NP40 Cell Lysis Buffer (Thermo Fisher

579   Scientific, MA, US) and quantified by Bradford method. Rat heart tissue was pre-

580   lysed using TissueLyser II (Qiagen, Hilden, Germany) before protein lysis buffer

581   addition. Protein samples were subjected to SDS-PAGE (10% acrylamide) and

582   electrotransferred onto PVDF membranes. After blocking with 5% non-fat dry

583   milk dissolved in Tris-buffered saline containing 0.1% Tween-20 (TTBS) for 1 h

584   at 37ºC, membranes were probed overnight at 4ºC with anti-CRF-R2 (Novus, CO,

585   US), anti-tubulin (Merck-Sigma-Aldrich, MI, US), anti-HMOX1 (Cell Signaling,

586   MA, US), anti-AIFM1 (Cell Signaling, MA, US) and anti-GAPDH (Sigma-Aldrich,

587   MI, US), in TTBS with 1% of BSA. After washing, membranes were incubated for

588   45 min at room temperature with a horseradish peroxidase conjugated with anti-

589   IgG (Cell Signaling, MA, US). Detection was performed in the ImageQuant LAS

590   4000 mini (GE Healthcare, IL, US). Images were analyzed with ImageJ software.

591   **Statistical analysis**

592   Analyzes were performed with GraphPad (GraphPad Software, Inc., CA, US),

593   using Shapiro-Wilk as normality test. For normally distributed variables we used

594   the Ordinary one-way ANOVA, and we performed the multiples comparisons

595   using t test without correction (Fisher's LSD test). We also used the non-

596   parametric test Kruskal-Wallis with multiple comparisons corrected by Dunn's

597   Test for non-normally distributed variables. Values were subjected to Log-

598   transformation to represent numerical features in the dataset to have a mean of 0

599   and a variance of 1 and to express data as "Relative gene/miR expression (Log

600 Fold Change)". The outliers were removed based on results of QuickCalcs, an

601 online tool of Graphpad. Results are presented as the mean ± SEM.

602

603 **AUTHOR CONTRIBUTIONS**

604 Conceptualization, A.H., and T.S.; funding acquisition, A.H., A.O., E.C.-S, and

605 T.S.; investigation, I.M.-G., E.C.-S., I.G.-O, E.G.-C, A.O., and T.S.; methodology,

606 I.M.-G., E.C.-S., I.G.-O, M.M.B., N.D., A.G., M.F.-V., A.H., and T.S.; project

607 administration, A.O., T.S.; writing—original draft, I.M.-G. and T.S.; and

608 writing—review and editing, N.D., M.C.-L, M.F.-V., A.G., D.-R.A., A.O., A.H.,

609 and T.S.

610

611 **CONFLICT OF INTEREST**

612 The authors declare that the research was conducted in the absence of any

613 commercial or financial relationships that could be interpreted as a potential

614 conflict of interest.

615

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629

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793

794

795 **TABLE**

796

797 **TABLE 1.** Data summary (mean ± SEM) of hemodynamic parameters evaluated in rats 1 week

798 after surgery in the following experimental group: Sham, "I/R", and "I/R + Ucn-2". LVdD is for

799 left ventricle diastolic diameter; LVEF: left ventricle ejection fraction; LVEdV: left ventricle end-

800 diastolic volume; LVFS: left ventricle fractional shortening; LVEsV: left ventricle end-systolic

801 volume. "*" and "#" indicate significance at $p < 0.05$ in Sham vs I/R and I/R vs IR+Ucn2,

802 respectively.

803

	LVEdV (ml)	LVEsV (ml)	LVdD (mm)	LVEF (%)	LVFS (%)
Sham (n=10)	0.42 ± 0.02	0.12 ± 0.01	5.91 ± 0.18	71.84 ± 1.23	19.98 ± 1.90
I/R (n=12)	0.50 ± 0.03[*]	0.20 ± 0.02[*]	6.85 ± 0.27[*]	60.16 ± 2.04[*]	16.77 ± 1.1[*]
I/R + Ucn-2 (n=15)	0.40 ± 0.02[#]	0.13 ± 0.01[#]	6.46 ± 0.16	66.43 ± 0.68[*,#]	18.16 ± 3.20

804
805
806
807 **FIGURE LEGENDS**

808

809 **FIGURE 1.** I/R increases of circulating urocortin-2 level and the expression of

810 **CRF-R2.** (A) Bar graph shows the concentration of circulating Ucn-2 in serum of

811 rats from sham, I/R 1 week (I/R 1w) and 6 weeks (I/R 6w) after surgery (n = 7-11).

812 (B) Plot of western blot and bar graph summarizing the expression of CRF-R2

813 expression and tubulin in rats' heart from sham, I/R (1 and 6 weeks) after surgery

814 (n = 8). Values are means ± SEM. "**" and "***" indicate significance at $p < 0.01$

815 and $p < 0.001$, respectively.

816 **FIGURE 2.** **Urocortin-2 improves contractility and prevents I/R-induced**

817 **fibrosis.** (A) Representative M-mode echocardiographic images evaluated 1

818 week after the intervention in Sham, I/R rats, and in rats infused with 150 μg/Kg

819   Ucn-2 (I/R + Ucn2). (B) Representative *in vivo* cardiac magnetic resonance images

820   taken from I/R and I/R + Ucn-2 rats. Gadolinium was used as contrast. The fibrotic

821   area is delimited by yellow lines. (C) Bar graph showing summary data of fibrotic

822   areas in I/R and I/R + Ucn-2 rats. (D) Panel shows representative Masson's

823   trichrome staining of transverse heart sections from I/R and I/R + Ucn-2. Healthy

824   tissue is stained by red, while fibrotic tissue in the infarcted zone is stained in

825   blue. (E-H) Bar graphs show the effect of Ucn-2 on the expression of pro-fibrotic

826   genes, collagen I (*Col-I*, E), collagen III (*Col-III*, F), Transforming growth factor $\beta1$

827   (*Tgf-$\beta1$*, G), and *Tgf-$\beta2$* (H), examined in the risk zone of the infarcted hearts 1

828   week after surgeries. Samples were from "Sham"; I/R and I/R + Ucn-2. Gene's

829   relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to

830   the internal control β-actin. Data are relative expression of Log fold change of

831   means ± SEM (n = 4-8). "*", "**", "***" and "****" indicate significance at $p < 0.05$,

832    $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

833   **FIGURE 3**. **Urocortin-2 attenuates the release of LDH and apoptosis.** (A) Bar

834   graph shows the level of LDH in the serum of rats from Sham, I/R and I/R + Ucn-

835   2, 24 h after surgery. (B) Representative snapshot of Tunnel staining (green) in

836   adult heart section from rats of the three experimental groups. Top: images taken

837   with a 10X objective, scale bar = 1 mm. Bottom: images cropped from upper ones.

838   (C) Representative images of heart sections stained for detection of cleaved

839   caspase 3 (upper panel) captured with a 40X objective, scale bar = 100 μm. Lower

840   panel shows merge images of heart's section stained with caspase 3 in red, wheat

841 germ agglutinin (WGA) in green and DAPI in blue used for nuclear staining. (D)

842 Representative images of annexin V (green) staining in adult cardiac myocytes

843 cells. Images are from untreated cells (control), and from cells exposed to I/R (30

844 min/24 h each) ± Ucn-2 (30 nM). Image were taken with a 20X objective, scale bar

845 = 100 μm. (E, F) Summary data showing the percentage of unstained live cardiac

846 myocytes, and annexin V-labeled cells related to control. Values are means ± SEM

847 (n = 4-6). "*", "**", and "***" indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$,

848 respectively.

849 **<u>FIGURE 4.</u> miRNAs expression in serum and heart tissue of STEMI patients,**

850 **and in rat cardiac ventricle risk zone.** (A) Bar graph summarizing microarray

851 results indicating release of circulating miRNAs examined in the serum of STEMI

852 patients 3-6 h after primary percutaneous coronary intervention. (B) Bar graph

853 shows the detection of selected miRNAs in the atrium of ischemic patients with

854 heart failure. ΔCt represents the level of Ct of miRNAs compared to the

855 endogenous control. Values are means ± SEM (n = 8-10). (C-H) Bar graphs show

856 the expression of miR-29a (C), miR-103 (D), miR-133 (E), miR-339-5p (F), miR-

857 423_1 (G) and miR-451_1* (H) examined in the risk zone of the infarcted heart of

858 sham, I/R and I/R+Ucn-2, 24 h and 1 week after surgery. Relative expression

859 levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the

860 expression of the endogenous control miRTC1. Values are relative expression of

861 Log fold change of means ± SEM (n = 4-6). "*", "**", "***" and "****" indicate

862 significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

863   <u>**FIGURE 5**</u>**. miR-29a and miR-451_1* are predicted to target apoptosis-related**

864   **genes.** (A) PANTHER analysis showing the predicted pathways go be targeted

865   by miR-29a, miR-103, miR-133, miR-339-5p, miR-423_1 and miR-451_1*. The pink

866   and grey slice in the pie chart highlight predicted genes related to apoptosis and

867   fibrosis. (B) Number of predicted target genes for each miRNA. (C-E) Graphs

868   show of the expression of 76 dysregulated genes examined using samples (n = 4)

869   from NRVM exposed to I/R (C), and from NRVM transfected with mimics of

870   miR-29a (D) and miR-451_1* (E) in I/R. Inserts show numbers and percentage of

871   upregulated and downregulated genes in each condition.

872   <u>**FIGURE 6.**</u> **miR-29a regulates apoptotic and fibrotic genes under I/R, and**

873   **signaling pathway of miR-29a regulation by Ucn-2.** (A-F) Bar graphs showing

874   the expression of *Apaf-1*, *Aifm-1*, *Bcl-2*, *Cycs*, *Hmox-1* and *Mapk-8*, in control, in

875   non-transfected NVRM under I/R (orange), and in NRVM transfected with miR-

876   29a (red) and 451_1* (green). (G, H) Bar graphs showing the expression of *Col- I*

877   and *Col-III* in similar conditions as in above. (I) Bar graph shows the expression

878   of miR-29a examined in untreated NRVM "Control", in NRVM under "I/R", in

879   NRVM treated with Ucn-2 (10 nM) before reperfusion "I/R + Ucn-2", and in

880   NRVM pretreated with Astressin (1 μM) to inhibit CRF-R2 "I/R+Ucn-2+Ast", H89

881   (1 μM) to inhibit PKA "I/R+Ucn-2+H89", ESI-05 (10 μM) to block Epac2 "I/R+Ucn-

882   2+ESI", and PD 098059 (5 μM) to inhibit ERK1/2 "I/R+Ucn-2+PD". Ucn-2 was

883   added before reperfusion. Relative expression levels were calculated using the

884     $2^{-\Delta\Delta Ct}$ method after normalization with the expression of the endogenous control

885 β-actin for genes and miRTC1 for miR-29a. Values are expressed as relative

886 expression of Log fold change of means ± SEM (n = triplicate of 3-4 cell culture).

887 "*", "**", and "****" indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.0001$,

888 respectively.

889 **FIGURE 7. Expression of apoptotic genes and protein I/R rat model.** (A-D) Bar

890 graphs showing the relative expressions of *Hmox-1*, *Aifm-1*, *Apaf-1*, and *Cycs*

891 calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with the expression of the

892 endogenous control β-actin. (E-G) Representative immunoblots and summary

893 data of protein expression of HMOX-1, AIFM-1, and CYCS assessed by western

894 blots and normalized to their corresponding GAPDH expression. GAPDH blots

895 in 7F and 7G are the same. Samples were from sham, I/R, and I/R + Ucn-2 rats,

896 and were processed 24 h and 1 week after surgery. Values are from n = 3-4. "*",

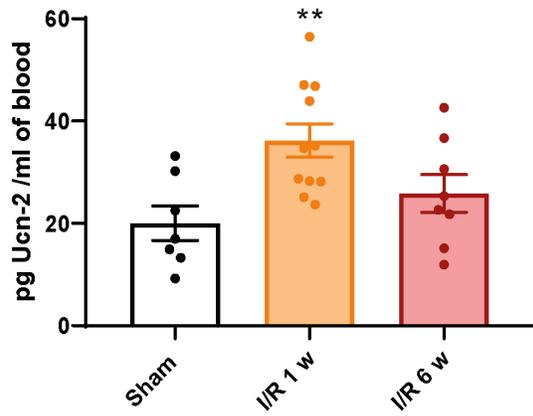897 "**" and "***" indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

898 **FIGURE 8. Expression of apoptotic genes in left ventricles of ischemic patients**

899 **with heart failure.** (A) Bar graph showing the expression of *Hmox-1*, *Mapk-8*,

900 *Cycs*, *Apaf-1*, *and Aifm-1*, in ventricle biopsies from ischemic patients with heart

901 failure, as compared to a healthy ventricle. (B-F) Graphs showing linear

902 regression analysis using the percentage of the left ventricular ejection fraction

903 (LVEF) vs the expression of *Hmox-1*, *Mapk-8*, *Cycs*, *Apaf-1*, *and Aifm-1*. Relative

904 expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization

905 with the expression of the endogenous control β-actin. Values are expressed as

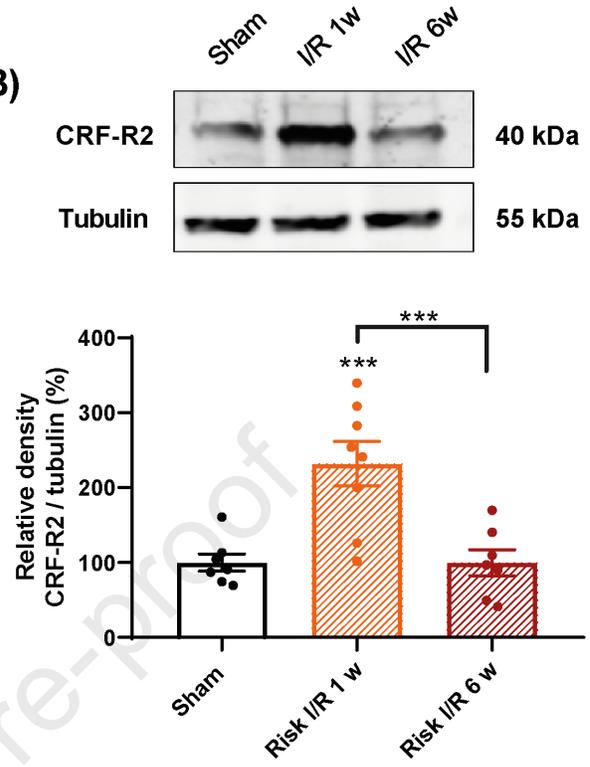906   relative expression of Log fold change of means ± SEM (n = 7). "*", "**" and "****"

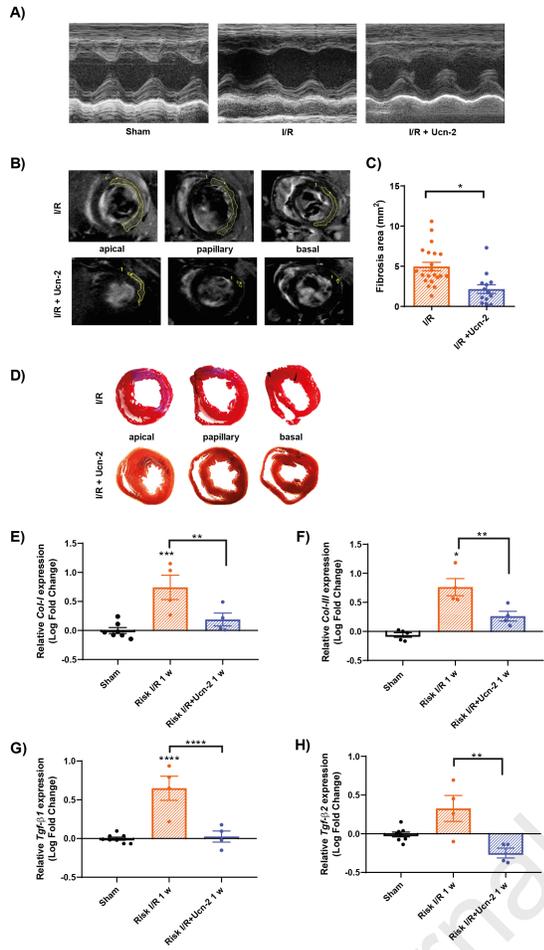907   indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.0001$ respectively.
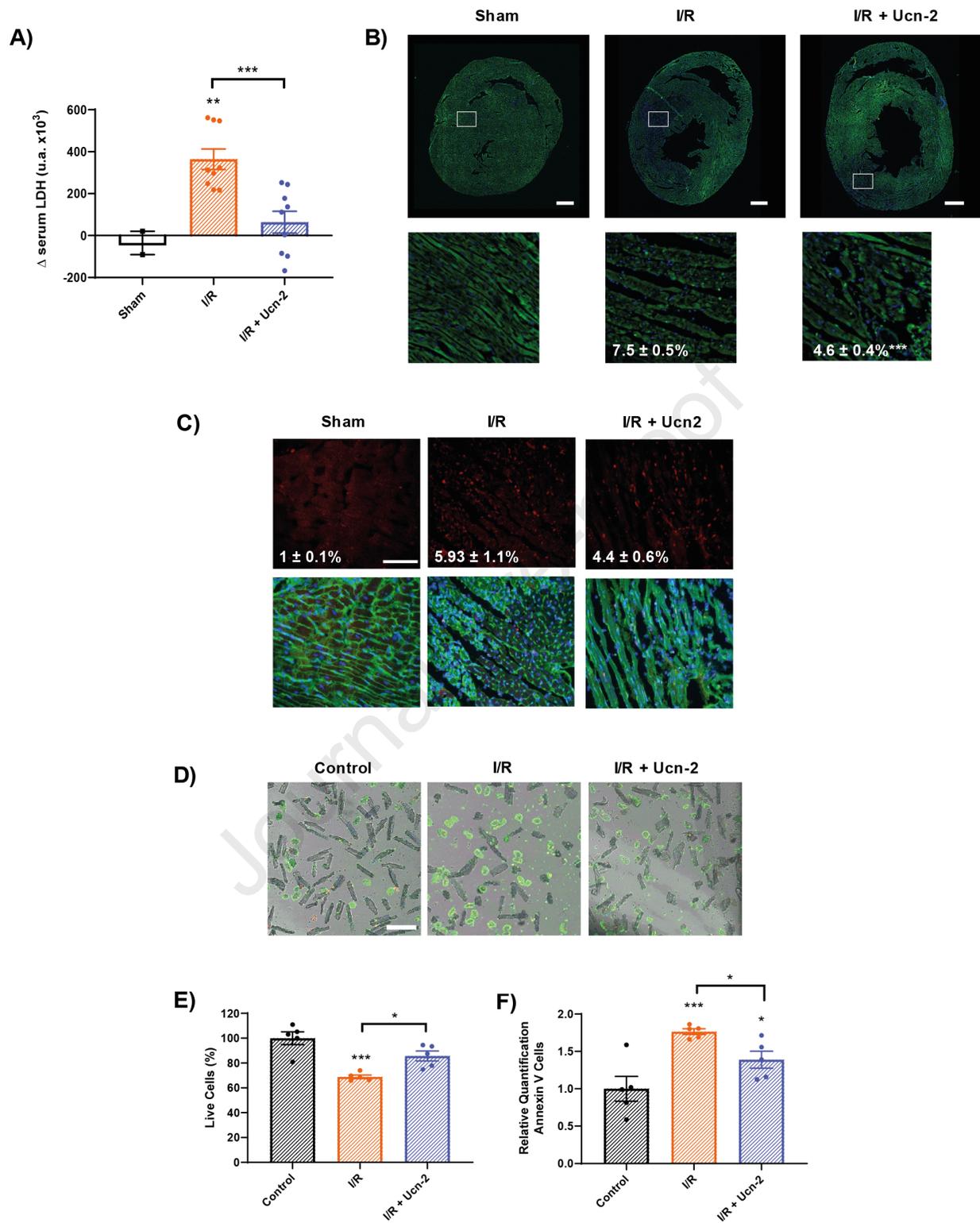
A)

B)

A)

Sham   I/R   I/R + Ucn-2

B)

I/R

apical   papillary   basal

I/R + Ucn-2

C)

Fibrosis area (mm²)

*

I/R   I/R +Ucn-2

D)

I/R

apical   papillary   basal

I/R + Ucn-2

E)

Relative *Col-I* expression (Log Fold Change)

**

***

Sham   Risk I/R 1 w   Risk I/R+Ucn-2 1 w

F)

Relative *Col-III* expression (Log Fold Change)

**

*

Sham   Risk I/R 1 w   Risk I/R+Ucn-2 1 w

G)

Relative *Tgf-β1* expression (Log Fold Change)

****

****

Sham   Risk I/R 1 w   Risk I/R+Ucn-2 1 w

H)

Relative *Tgf-β2* expression (Log Fold Change)

**

Sham   Risk I/R 1 w   Risk I/R+Ucn-2 1 w

A)

B) Sham   I/R   I/R + Ucn-2

7.5 ± 0.5%   4.6 ± 0.4% ***

C) Sham   I/R   I/R + Ucn2

1 ± 0.1%   5.93 ± 1.1%   4.4 ± 0.6%

D) Control   I/R   I/R + Ucn-2

E)   F)

A)

Targeted genes of candidates miRNAs

Apoptosis

Fibrosis

Legend (right):
- Co-activator or corepressors
- Jun-N terminal kinase
- Rsmads
- Ras-GDP
- Ras-GTP
- Smad anchor for receptor activation
- Smad ubiquitination regulatory factors
- TGF-β receptor type I
- TGF-β receptor type II
- TGF-β receptors
- TGF-β activated kinase-1
- Transforming growth factor b
- P38 mitogen activated protein kinase
- P42/p44 MAPKs
- Partner Smads

Legend (left):
- Activating transcription factor
- Apoptosis inducing factor
- Bcl-2 antagonist killer
- Bcl-2associated X protein
- Bcl-2–w
- Bim
- Caspase 8
- Cellular inhibitor apoptosis protein 1-2
- Fos
- Germinal center kinase
- Inhibitor of κ–β-kinase
- Jun kinase
- MAPK7
- MAPK
- MCL1
- NFκβ
- PI3K
- Protein activator of interon.induced protein kinase
- PKC
- Receptor interacting protein
- SAPK/ERK kinase 1
- Second mitocondria-derived activator of caspase
- Translation initiation factor 2-α
- Tumor necrosis factor receptor 1
- X-linked inhibitor of apoptosis
- Protein kinase B

B)

	Targeted Apoptosis Genes	Targeted Fibrosis Genes
miR-29a	16	14
miR-103	5	4
miR-133	15	6
miR-339-5p	3	3
miR-423_1	1	-
miR-451_1*	17	18

C) I/R

Relative genes expression (Log fold change)

	n	(%)
Upregulated genes	56	73.69
Downregulated genes	20	26.31

D) I/R + miR-29a mimic

Relative genes expression (Log fold change)

	n	(%)
Upregulated genes	20	26.31
Downregulated genes	56	73.69

E) I/R + miR-451_1* mimic

Relative genes expression (Log fold change)

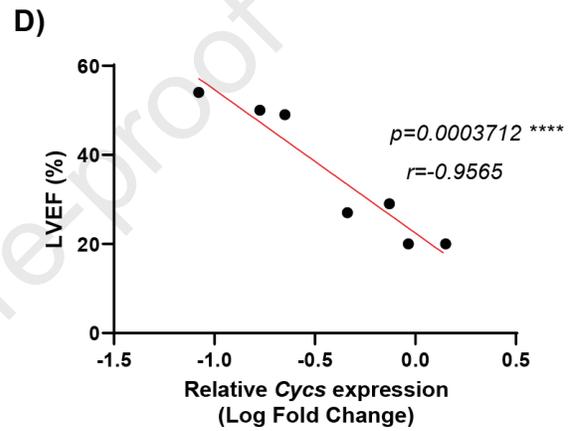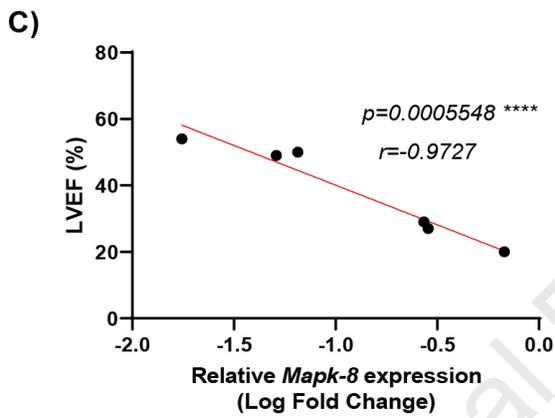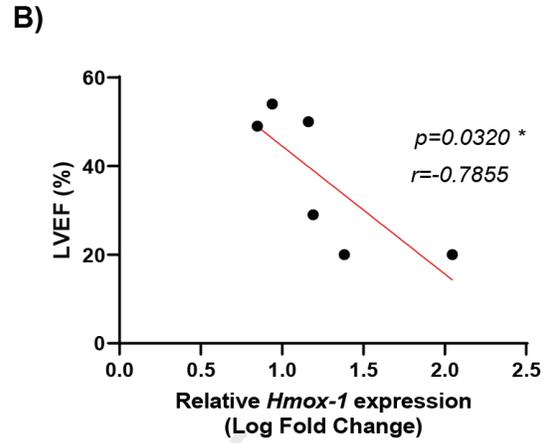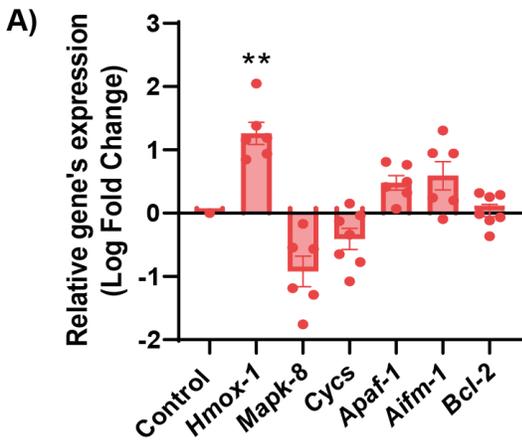|	n	(%)
Upregulated genes	27	35.52
Downregulated genes	49	64.48
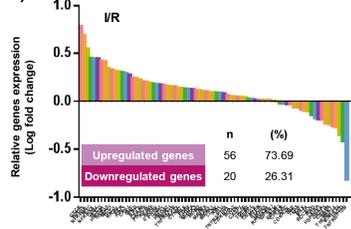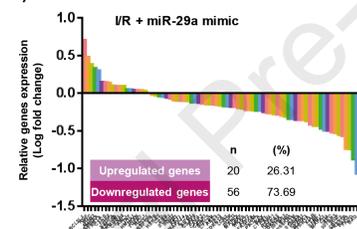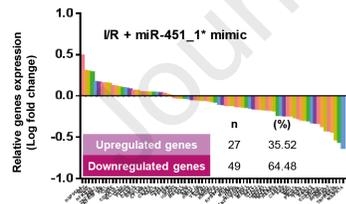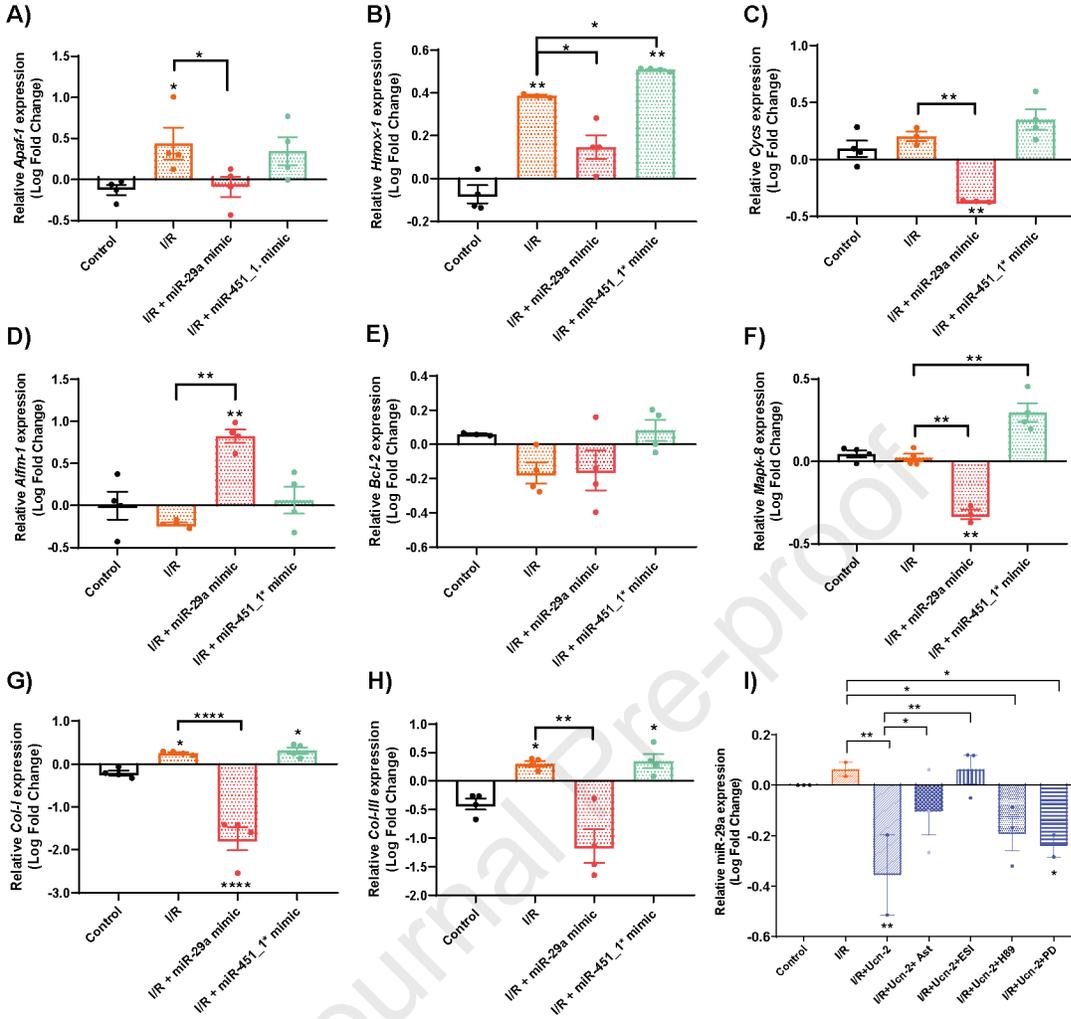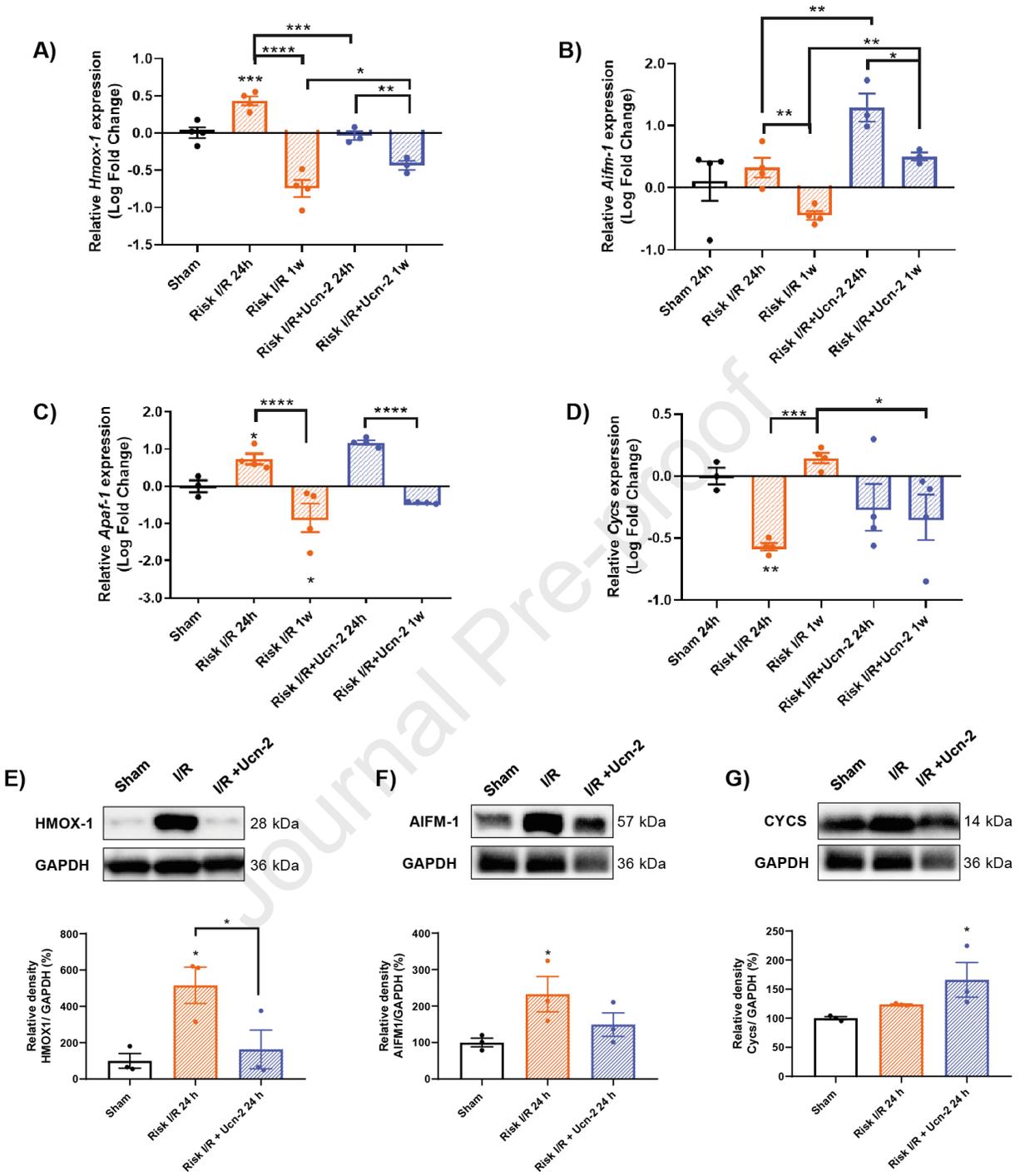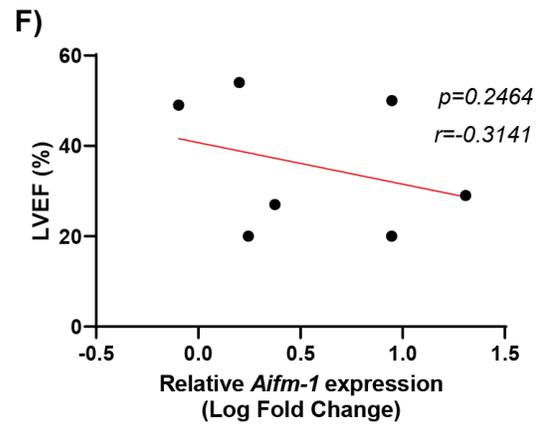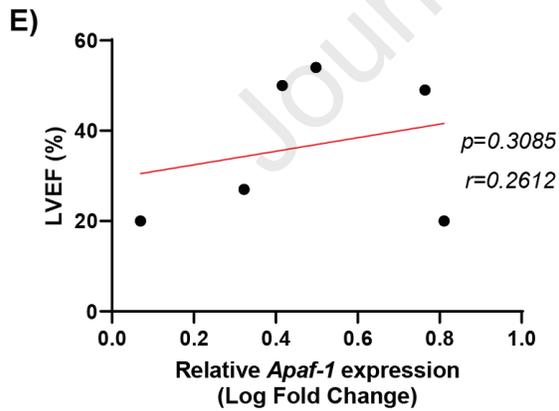
eTOC Synopsis

Mayoral-González et al. used heart samples from ischemic patients and animal models to demonstrate the sustained protective role of Urocortin-2, which induced changes in the expression of miR-29a and specific genes associated with cardiomyocyte death and fibrosis, critical events in the progression of adverse cardiac remodelling towards heart failure.