Myocardial IGF2R is a critical mediator of inflammation and fibrosis after ischemia-reperfusion injury

Zhichao Wu[1], Wei Huang[1], Xingyu He[1], Suchandrima Dutta[2], Christian Paul[1], Guo-Chang Fan[3], Onur Kanisicak[1], Meifeng Xu[1], Jialiang Liang[1,*], Yigang Wang[1,*]

1 Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati, Cincinnati, OH, USA, 45267

2 Department of Internal Medicine, College of Medicine, University of Cincinnati, Cincinnati, OH 45267

3 Department of Pharmacology and Systems Physiology, College of Medicine, University of Cincinnati, Cincinnati, OH, USA, 45267

* Correspondence to: Jialiang Liang (liangjl@ucmail.uc.edu), Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, Ohio, 45267, USA. Yigang Wang (yigang.wang@uc.edu), Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, Ohio, 45267, USA.

ABSTRACT

Ischemia-reperfusion (I/R) injury is a common occurrence in various surgical procedures used to treat heart diseases. However, the role of insulin-like growth factor 2 receptor (IGF2R) during the process of myocardial I/R remains unclear. Therefore, this study aims to investigate the expression, distribution, and functionality of IGF2R in various I/R-associated models (such as reoxygenation, revascularization, and heart transplant). Loss-of-function studies (including myocardial conditional knockout and CRISPR interference) were performed to clarify the role of IGF2R in I/R injuries. Following hypoxia, IGF2R expression increased, but this effect was reversed upon restoration of oxygen levels. Loss of myocardial IGF2R was found to enhance the cardiac contractile functions, and reduced cell infiltration or cardiac fibrosis of I/R mouse models compared to the genotype control. CRISPR-inhibition of IGF2R decreased cell apoptotic death under hypoxia. RNA sequencing analysis indicated that myocardial IGF2R played a critical role in regulating the inflammatory response, innate immune response, and apoptotic process following I/R. Integrated analysis of the mRNA profiling, pulldown assays, and mass spectrometry identified granulocyte-specific factors as potential targets of myocardial IGF2R in the injured heart. In conclusion, myocardial IGF2R emerges as a promising therapeutic target to ameliorate inflammation or fibrosis following I/R injuries.

INTRODUCTION

Ischemia-reperfusion (I/R) injury is a well-known phenomenon that occurs in various surgical procedures to treat heart diseases, such as percutaneous coronary intervention for acute myocardial infarction, heart transplantation, and coronary artery bypass grafting.[1] While several pharmacological interventions have been investigated to improve I/R injury, no single therapy can effectively target the multiple pathways involved in this complex process.[2] Eventually, the activation of irreversible cell death pathways after I/R can contribute to the development of heart failure. Understanding the molecular and cellular mechanisms underlying I/R injury is critical for refining the existing surgical approaches to prevent or mitigate their adverse effects on the heart.

Currently, most of the drugs being evaluated for their cardioprotective effects are derived from the cardioprotective signal transduction pathways.[3, 4] The activation of prosurvival pathways (such as PI3K/AKT, JAK/STAT, and ERK) modulated by the insulin-like growth factor (IGF) system is essential for cardiac homeostasis including cell growth or death and regulation of energy metabolism.[5-7] Previous

54 studies have established the homology between insulin, IGF1, and IGF2, as well as
55 elucidated the interactions among the insulin receptor (INSR), IGF1 receptor (IGF1R),
56 and IGF2 receptor (IGF2R).[8-10] As the administration of insulin, IGF1, IGF2, and
57 other growth factors gains increasing interest as a potential treatment for myocardial
58 I/R,[11-13] investigating the receptors of these factors could provide valuable insights
59 into the role of the IGF system in cardioprotection.

60 It is well-known that canonical downstream of IGF1R such as PI3K/AKT can
61 maintain outer mitochondrial membrane potential and inhibit glycogen synthase
62 kinase-3β, thereby protecting cells against necrosis triggered by I/R.[14-16] Recent
63 research has shown that inhibiting IGF2R signaling promotes cardiomyocyte
64 proliferation and reduces hypoxia- or TNFα-induced cell death,[17-19] while the role of
65 IGF2R signaling in I/R remains largely unknown. With broad expression across many
66 cell types, IGF2R is a versatile receptor that can bind multiple ligands (including
67 IGF2 and M6P-modified proteins) and plays a critical role in intracellular transport,
68 shuttling between the trans-Golgi network, endosomes, and plasma membrane.[20, 21]
69 Therefore, disruption of the Golgi-to-lysosome transport by loss of IGF2R can lead to
70 reduced lysosomal activity, resulting in the accumulation of misfolded proteins and
71 increased production of reactive oxygen species due to dysfunction in both autophagy
72 and mitophagy pathways.[22, 23] Given its multifunctional nature, the effects of IGF2R
73 on cell viability appear to be cell type-specific and context-dependent under stress
74 conditions. In this study, the loss-of-function models were used to investigate the role
75 of IGF2R in myocardial I/R injuries.

76 **RESULTS**

77 **Dynamic expression of IGF2R during myocardial I/R**

78 The expression of IGF2R in mouse hearts exhibited an initial increase
79 following myocardial ischemia (MI) and reperfusion as compared to the sham
80 operation but subsequently decreased to baseline levels after a 6-hour reperfusion
81 period (**Fig.1A**). In addition to cardiomyocytes, IGF2R was expressed in non-CM
82 cells located in the interstitial space of reperfused heart (**Fig.1B**). The single-cell
83 RNA-seq dataset (Tabula Muris)[24] also provides evidence that IGF2R is expressed
84 constitutively across multiple cell types within the heart (**Fig.1C**). To investigate the
85 precise role of myocardial IGF2R in I/R, we generated conditional knockout (cKO)
86 models by crossing Cre-inducible $Igf2r^{fl/fl}$ mice with $Myh6^{MerCreMer}$ mice and treating

87 the resultant offspring with tamoxifen (**Fig.1D**), while *Igf2r*$^{fl/fl}$ litters were used as the
88 control group (Ctrl). IGF2R gene expression was significantly decreased in cKO as
89 confirmed by PCR and western blotting (**Fig.1E-F**).

90 **Loss of myocardial IGF2R improves heart function of I/R models**

91 Subsequently, two different types of IR injury were employed to assess the
92 effect of cKO on heart function. In a form of warm I/R (**Fig.2A**), the heart function of
93 Ctrl or cKO mice was analyzed by echocardiography after LAD ligation surgery. At 1
94 week after I/R, there was no difference in the ejection fraction (EF) between Ctrl and
95 cKO (**Fig.2B**). The heart function of cKO mice demonstrated an improvement at 4
96 weeks post-I/R when compared to Ctrl mice, with increases in EF, fractional
97 shortening, and interventricular septal (**Fig.2C-D**). In addition, heterotopic cardiac
98 transplant models were established to mimic cold I/R injury (**Fig.2E**). Following
99 transplantation within 12-hour preservation in cold HTK solutions, there was no
100 difference in re-beating time between the control group and the cKO donor hearts
101 (**Fig. 2F**). Although the re-beating time of cKO hearts was shorter than that of the
102 control group after 24-hour preservation in cold HTK solutions, both grafts had a
103 dramatically decreased median survival time (**Fig. 2G**).

104 All Ctrl and cKO donor hearts survived after 6-hour preservation without a
105 significant difference, but ~60% of cKO hearts exhibited a significantly longer
106 median survival time than Ctrl hearts after 12-hour preservation (**Fig.2G**), suggesting
107 a potential protective effect of cKO against ischemic injury. Therefore, on day one
108 post-surgery, a catheter-based approach was used to evaluate the left ventricular (LV)
109 pressure and contractility of Ctrl or cKO hearts that underwent 12-hour preservation
110 (**Fig.2H-I**). The LV systolic pressure and heart rate were increased in cKO donor
111 hearts as compared with the control, while there was no significant difference in the
112 LV end-diastolic pressures. The +dp/dt max and the -dp/dt max were rapidly raised in
113 cKO donor hearts, reflecting an improved contractile function. Furthermore,
114 recipients with cKO hearts demonstrated a significant decrease in serum levels of
115 intracellular enzymes, including creatine kinase and lactate dehydrogenase, when
116 compared to the control group (**Fig.2J**).

117 **Deletion of myocardial IGF2R alters transcriptional profiles in response to I/R**

118 RNA sequencing (RNA-seq) was performed to analyze the transcriptome of
119 cKO and Ctrl hearts at baseline and after 12-hour preservation and transplantation

120 (I/R). Principal component analysis of the normalized datasets showed that the
121 transcriptional profiles of the uninjured cKO and Ctrl hearts were similar, while the
122 cKO hearts exhibited distinct expression patterns compared to the other samples
123 following I/R (**Fig.3A**). To gain a deeper understanding of the underlying mechanism
124 of IGF2R, we analyzed the differentially expressed genes in cKO and Ctrl hearts
125 subjected to the I/R condition.

126 We identified 3,909 significant genes (*padj*<0.05) differentially expressed in
127 cKO and Ctrl hearts following I/R, and used hierarchical clustering analysis to group
128 these genes into distinct clusters (**Fig.3B**). After I/R, clusters a and b showed up-
129 regulated gene expression in cKO hearts, while clusters c and d exhibited up-
130 regulated gene expression in Ctrl hearts. At baseline, both cKO and Ctrl hearts
131 displayed similar patterns of up-regulated gene expression in clusters b and c, and
132 down-regulated gene expression in clusters a and d. Gene ontology (GO) analysis was
133 used to identify the over-representation of GO terms (biological processes) in the gene
134 lists of different clusters (**Fig.3C**). Genes involved in DNA repair, cell cycle
135 regulation, and autophagy were predominantly represented in clusters a and b, while
136 clusters c and d were enriched for genes related to inflammatory response, innate
137 immune response, and apoptotic process. The top 20 GO terms showed that the cKO
138 of IGF2R significantly attenuated innate immune responses, including cytokine
139 production, leukocyte migration, and cell adhesion, while promoting cell cycle
140 progression and self-healing processes. (**Fig.3D**).

141 We also performed Gene Set Enrichment Analysis (GSEA) on a ranked list of
142 significant genes to identify enriched gene sets and biological pathways associated
143 with the observed gene expression changes (**Fig.3E**). The results of the gene set
144 distribution and enrichment score using GSEA confirmed that the Ctrl hearts were
145 enriched for GO terms related to inflammatory response, innate immune response,
146 and chemotaxis, whereas the cKO hearts showed enrichment for the mitotic cell cycle
147 process except for autophagy. The cKO of IGF2R resulted in the activation of genes
148 involved in cell growth pathways, which supports the established concept that IGF2R
149 functions as a growth-inhibitory gene.[21, 25].

150 **Identification of IGF2R-associated proteins from reperfused myocardium**

151 Although the innate immune response is known to play a critical role in the
152 pathogenesis of myocardial I/R injury,[26, 27] the precise mechanism by which IGF2R

153 participates in this process is still unknown. Therefore, we attempted to identify the
154 IGF2R targets by proteomic analysis of lysed tissues obtained from heart grafts using
155 pull-down assays on day 1 post-I/R (**Fig.4A**). To this end, we selectively excised
156 protein bands from the control sample, which were missing from the cKO sample at
157 equivalent molecular weights, and performed mass spectrometry analysis. The lists of
158 identified proteins were compared with the RNA-seq results of the down-regulated
159 (log2fc<-1, padj<0.05) or up-regulated (log2fc>1, padj<0.05) genes (**Fig.4B**). Notably,
160 the consistent down-regulation of *Actb*, *Ngp*, *Chil3 (Chi3l3)*, *S100a9*, and *Ltf* gene in
161 cKO heart tissue at both transcriptomic and translational levels highlighted their
162 potential role as therapeutic targets of IGF2R. The single-cell RNA-seq dataset
163 (Tabula Muris)[24] also reveals that these genes are expressed in granulocytes,
164 granulocytopoietic cells, and monocytes in bone marrow, but not in the native heart
165 (**Fig.4C**). At 4 weeks post I/R injury, the Ctrl heart exhibited a substantial increase in
166 cell infiltration with visible nuclei, while the cKO heart demonstrated a significant
167 reduction in cell infiltration (**Fig.4D**). Furthermore, Masson's trichrome staining
168 showed that the fibrosis area in the cKO heart was smaller than that in the Ctrl heart
169 (**Fig.4E**).

170 **Hypoxia-induced activation of IGF2R leads to apoptotic cell death**

171 IGF2R was expressed in H9C2 cardiomyoblasts, and *in vitro* model of
172 hypoxia-reoxygenation was used to mimic I/R injury. As compared to the normoxic
173 condition, the IGF2R protein level was increased after hypoxia for 3 hours but slightly
174 decreased after reoxygenation (**Fig.5A-B**). It is known that IGF2R shuttles constantly
175 between intracellular organelles (~90% of the total IGF2R protein) and the cell
176 surface membrane.[9, 25] Labeling with wheat germ agglutinin indicated that IGF2R was
177 primarily localized in the intracellular organelles of H9C2 cells (**Fig.5C**).
178 Interestingly, under hypoxia, the intracellular IGF2R appeared to diffuse from the
179 perinuclear region toward the plasma membrane, resulting in a dispersed cytoplasmic
180 distribution. Following hypoxia-reoxygenation, the immunoblot analysis of protein
181 fractions revealed that IGF2R was highly expressed in the intracellular membrane
182 fraction, indicating membrane attachment rather than cytoplasmic solubility (**Fig.5D**).

183 A doxycycline (Dox)-inducible CRISPR-interference (CRISPRi) system
184 (pHAGE-TRE-dCas9-KRAB)[28] was established to determine whether the increased
185 IGF2R protein level was due to its promoter transcription activation (**Fig.5E**). The

186 IGF2R protein expression in H9C2 cells was decreased by Dox-induced CRISPRi
187 under normal or hypoxia-reoxygenation conditions (**Fig.5F**). Hypoxia caused a
188 significant increase in the level of cleaved caspase3 (active form), which was
189 attenuated by Dox treatment. Although the cleaved caspase3 level progressively
190 decreased after reoxygenation (to the normoxic level), CRISPRi of IGF2R did not
191 affect cell death during this period.

192 **DISCUSSION**

193 Although IGF2R is known to be multifaceted, its role in the myocardial I/R
194 response remains poorly characterized. Here, we investigated the role of IGF2R in
195 different models of myocardial I/R injuries. We observed the dynamic expression of
196 IGF2R during myocardial I/R and found that cardiac-specific depletion of IGF2R
197 improved heart dysfunction and reduced cell death. Mechanistic studies revealed that
198 IGF2R plays a role in the inflammatory response and fibrosis by interacting with
199 immune cell-derived proteins. Our findings suggest that IGF2R could be an important
200 therapeutic target for I/R injury and warrant further exploration of its function in
201 cardiac myocytes.

202 Our results showed that IGF2R expression was upregulated in the ischemic
203 heart but returned to baseline levels following reperfusion. The dynamic expression of
204 IGF2R was also observed in renal macrophages during the I/R processes.[29] IGF2R is
205 expressed widely in both cardiac and non-cardiac cells, but whether its expression is
206 consistent or variable across different cell types in response to injuries remains to be
207 determined. To this end, single-cell RNA sequencing can be used to characterize the
208 expression of IGF2R across different cell types and heart disease models in future
209 studies. Further research is also needed to fully elucidate the potential transcriptional
210 or epigenetic mechanisms (such as DNA methylation, histone modifications,
211 microRNAs, long non-coding RNAs, and various transcription factors)[30-34] controlling
212 IGF2R expression in the context of I/R.

213 Our loss-of-function studies provided novel evidence that myocardial IGF2R
214 played a role in the immune responses following reperfusion or heart transplantation.
215 Depleting cardiac IGF2R expression resulted in a reduction of cell infiltration and
216 fibrosis in the heart tissue after reperfusion injury. The integrated analysis of the
217 transcriptome and proteome highlighted Ngp, Chil3, S100a9, and Ltf as potential
218 targets of IGF2R. Given the selective expression of these genes in bone marrow

219 granulocytes but not in normal cardiac cells, we reasoned that IGF2R could be a key
220 player in regulating the interplay between cardiomyocytes and granulocytes after
221 myocardial damage. Immune cell infiltration is a common response to stress signals in
222 the myocardium and is observed across various types of myocardial injuries.[35]
223 Neutrophils and other granulocytes can rapidly and massively infiltrate the site of
224 injury and release reactive oxygen species, proteolytic enzymes, and other cytotoxic
225 molecules that can cause further damage to the myocardium and contribute to the
226 development of cardiac fibrosis.[36, 37] Ngp, Chil3, S100a9, and Ltf are among several
227 proteins secreted by granulocytes that have been implicated in a variety of
228 pathological processes underlying heart disease, including inflammation, immune
229 response, and fibrosis.[38-42] Therefore, evidence suggests that IGF2R-mediated protein
230 capture may lead to activation of inflammation pathways in cardiomyocytes and
231 subsequent impairment of heart function following I/R injury. However, further
232 investigation is needed to elucidate the precise mechanisms involved.

233 Furthermore, our findings indicated that myoblasts subjected to hypoxia
234 experienced an increase in IGF2R expression, which was reversed upon
235 reoxygenation. This pattern was consistent with the expression trend of IGF2R
236 observed in heart tissue during the I/R processes. It is possible that the regulation of
237 IGF2R expression during hypoxia and reoxygenation is mediated by different
238 transcription factors or signaling pathways. However, previous studies investigating
239 the impact of hypoxia on the expression of the IGF2R gene have yielded conflicting
240 results. Some studies have shown increased expression of IGF2R during hypoxia in
241 myoblast cells,[17, 18] while others have found that hypoxia did not affect IGF2R
242 expression in ectoplacental cones.[43] Therefore, the effects of hypoxia on IGF2R
243 expression appear to be cell type-specific and involve multiple signaling pathways.
244 Aside from its overall gene expression level, the functionality of IGF2R is also
245 dependent on its intracellular localization.[44] The results of our study demonstrated that
246 hypoxia enhanced the intracellular trafficking of IGF2R from the perinuclear region
247 to the plasma membrane, and this effect was primarily attributed to membrane
248 attachment rather than cytoplasmic solubility.

249 To assess the therapeutic implications of specifically targeting IGF2R
250 expression, we utilized CRISPRi complex through the Dox-controlled transactivator
251 system to block the IGF2R promoter region in myoblasts. By enabling targeted gene
252 regulation, the CRISPRi technique offers remarkable specificity, versatility, and

253 reversibility, and presents a promising approach for treating a diverse range of
254 diseases.[45, 46] IGF2R expression was significantly eliminated by CRISPRi which was
255 found to attenuate cell death under hypoxic conditions, yet the molecular pathways
256 involved in this protective effect remain to be determined. Future studies using I/R
257 animal models will be also needed to determine the potential therapeutic effect of this
258 approach.

259 In summary, this study investigates the expression and function of IGF2R in
260 various models of myocardial I/R injury. We observed dynamic changes in IGF2R
261 expression and intracellular distribution in response to ischemic or hypoxic conditions.
262 Deletion of IGF2R resulted in improved heart function and reduced cell death,
263 indicating its involvement in myocardial I/R injury. RNA sequencing analysis
264 revealed that IGF2R deletion altered transcriptional profiles in response to I/R,
265 attenuating innate immune responses while promoting cell cycle progression and self-
266 healing processes. Proteomic analysis identified granulocyte-specific factors as
267 potential targets of IGF2R binding. Taken together, these findings suggest that
268 myocardial IGF2R plays an essential role in I/R injury and that its deletion may offer
269 a cardioprotective effect against inflammation or fibrosis. This study contributes to
270 our understanding of the pathogenesis of myocardial I/R injury and identifies IGF2R
271 as a potential therapeutic target for ischemic heart disease.

272 **ACKNOWLEDGMENTS**

273 This work was supported by the American Heart Association award
274 22TPA968781 (J.L.) and the National Institutes of Health (NIH) grants including
275 R01HL143490 and R01HL157456 (Y.W.). We would like to thank Dr. Randy Jirtle
276 and Dr. David Skaar (North Carolina State University) for providing the *Igf2r*$^{fl/fl}$ mice.

277 **DECLARATION OF INTERESTS**

278 The authors declare no competing interests.

279 **SUPPLEMENTAL METHODS**

280 All research protocols conformed to the Guidelines for the Care and Use of
281 Laboratory Animals published by the National Institutes of Health (National
282 Academies Press, eighth edition, 2011). All animal use protocols and experiments
283 conducted in this study were approved and oversight by the University of Cincinnati
284 Animal Care and Use Committee. Detailed methods are available in the supplemental
285 information.

286

287 **REFERENCES**

288 1. Eltzschig HK, Eckle T. Ischemia and reperfusion--from mechanism to
289 translation. *Nat Med*. 2011;17:1391-1401
290 2. Ibanez B, Heusch G, Ovize M, Van de Werf F. Evolving therapies for
291 myocardial ischemia/reperfusion injury. *J Am Coll Cardiol*. 2015;65:1454-
292 1471
293 3. Kleinbongard P, Heusch G. Extracellular signalling molecules in the
294 ischaemic/reperfused heart - druggable and translatable for cardioprotection?
295 *Br J Pharmacol*. 2015;172:2010-2025
296 4. Heusch G. Myocardial ischaemia-reperfusion injury and cardioprotection in
297 perspective. *Nat Rev Cardiol*. 2020;17:773-789
298 5. Laustsen PG, Russell SJ, Cui L, Entingh-Pearsall A, Holzenberger M, Liao R,
299 Kahn CR. Essential role of insulin and insulin-like growth factor 1 receptor
300 signaling in cardiac development and function. *Mol Cell Biol*. 2007;27:1649-
301 1664
302 6. Guo CA, Guo S. Insulin receptor substrate signaling controls cardiac energy
303 metabolism and heart failure. *The Journal of endocrinology*. 2017;233:R131-
304 r143
305 7. O'Sullivan JF, Leblond AL, Kelly G, Kumar AH, Metharom P, Buneker CK,
306 Alizadeh-Vikali N, Hristova I, Hynes BG, O'Connor R, Caplice NM. Potent
307 long-term cardioprotective effects of single low-dose insulin-like growth
308 factor-1 treatment postmyocardial infarction. *Circulation. Cardiovascular*
309 *interventions*. 2011;4:327-335
310 8. Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML,
311 Efstratiadis A. Mouse mutants lacking the type 2 igf receptor (igf2r) are
312 rescued from perinatal lethality in igf2 and igf1r null backgrounds. *Dev Biol*.
313 1996;177:517-535
314 9. El-Shewy HM, Luttrell LM. Insulin-like growth factor-2/mannose-6
315 phosphate receptors. *Vitamins and hormones*. 2009;80:667-697
316 10. De Meyts P, Whittaker J. Structural biology of insulin and igf1 receptors:
317 Implications for drug design. *Nat Rev Drug Discov*. 2002;1:769-783
318 11. Salminen PR, Dahle GO, Moen CA, Wergeland A, Jonassen AK, Haaverstad
319 R, Matre K, Grong K. Reperfusion therapy with low-dose insulin or insulin-
320 like growth factor 2; myocardial function and infarct size in a porcine model

321 of ischaemia and reperfusion. *Basic & clinical pharmacology & toxicology*.
322 2014;115:438-447
323 12. Zhang HX, Zang YM, Huo JH, Liang SJ, Zhang HF, Wang YM, Fan Q, Guo
324 WY, Wang HC, Gao F. Physiologically tolerable insulin reduces myocardial
325 injury and improves cardiac functional recovery in myocardial
326 ischemic/reperfused dogs. *J Cardiovasc Pharmacol*. 2006;48:306-313
327 13. White SJ, Chong JJH. Growth factor therapy for cardiac repair: An overview
328 of recent advances and future directions. *Biophys Rev*. 2020;12:805-815
329 14. Song HP, Zhang L, Dang YM, Yan H, Chu ZG, Huang YS. The
330 phosphatidylinositol 3-kinase-akt pathway protects cardiomyocytes from
331 ischaemic and hypoxic apoptosis via mitochondrial function. *Clinical and*
332 *experimental pharmacology & physiology*. 2010;37:598-604
333 15. Gomez L, Paillard M, Thibault H, Derumeaux G, Ovize M. Inhibition of
334 gsk3beta by postconditioning is required to prevent opening of the
335 mitochondrial permeability transition pore during reperfusion. *Circulation*.
336 2008;117:2761-2768
337 16. Yamashita K, Kajstura J, Discher DJ, Wasserlauf BJ, Bishopric NH, Anversa
338 P, Webster KA. Reperfusion-activated akt kinase prevents apoptosis in
339 transgenic mouse hearts overexpressing insulin-like growth factor-1. *Circ Res*.
340 2001;88:609-614
341 17. Chen WK, Kuo WW, Hsieh DJ, Chang HN, Pai PY, Lin KH, Pan LF, Ho TJ,
342 Viswanadha VP, Huang CY. Creb negatively regulates igf2r gene expression
343 and downstream pathways to inhibit hypoxia-induced h9c2 cardiomyoblast
344 cell death. *International journal of molecular sciences*. 2015;16:27921-27930
345 18. Lin KH, Kuo CH, Kuo WW, Ho TJ, Pai P, Chen WK, Pan LF, Wang CC,
346 Padma VV, Huang CY. Nfil3 suppresses hypoxia-induced apoptotic cell death
347 by targeting the insulin-like growth factor 2 receptor. *J Cell Biochem*.
348 2015;116:1113-1120
349 19. Chen Z, Ge Y, Kang JX. Down-regulation of the m6p/igf-ii receptor increases
350 cell proliferation and reduces apoptosis in neonatal rat cardiac myocytes. *BMC*
351 *cell biology*. 2004;5:15
352 20. Hille-Rehfeld A. Mannose 6-phosphate receptors in sorting and transport of
353 lysosomal enzymes. *Biochim Biophys Acta*. 1995;1241:177-194

354 21. Ghosh P, Dahms NM, Kornfeld S. Mannose 6-phosphate receptors: New
355 twists in the tale. *Nat Rev Mol Cell Biol*. 2003;4:202-212
356 22. Takeda T, Komatsu M, Chiwaki F, Komatsuzaki R, Nakamura K, Tsuji K,
357 Kobayashi Y, Tominaga E, Ono M, Banno K, Aoki D, Sasaki H. Upregulation
358 of igf2r evades lysosomal dysfunction-induced apoptosis of cervical cancer
359 cells via transport of cathepsins. *Cell Death Dis*. 2019;10:876
360 23. Pluvinage JV, Sun J, Claes C, Flynn RA, Haney MS, Iram T, Meng X,
361 Lindemann R, Riley NM, Danhash E, Chadarevian JP, Tapp E, Gate D,
362 Kondapavulur S, Cobos I, Chetty S, Pasca AM, Pasca SP, Berry-Kravis E,
363 Bertozzi CR, Blurton-Jones M, Wyss-Coray T. The cd22-igf2r interaction is a
364 therapeutic target for microglial lysosome dysfunction in niemann-pick type c.
365 *Sci Transl Med*. 2021;13:eabg2919
366 24. Tabula Muris C, Overall c, Logistical c, Organ c, processing, Library p,
367 sequencing, Computational data a, Cell type a, Writing g, Supplemental text
368 writing g, Principal i. Single-cell transcriptomics of 20 mouse organs creates a
369 tabula muris. *Nature*. 2018;562:367-372
370 25. Brown J, Jones EY, Forbes BE. Interactions of igf-ii with the igf2r/cation-
371 independent mannose-6-phosphate receptor mechanism and biological
372 outcomes. *Vitamins and hormones*. 2009;80:699-719
373 26. Timmers L, Pasterkamp G, de Hoog VC, Arslan F, Appelman Y, de Kleijn DP.
374 The innate immune response in reperfused myocardium. *Cardiovasc Res*.
375 2012;94:276-283
376 27. Arslan F, de Kleijn DP, Pasterkamp G. Innate immune signaling in cardiac
377 ischemia. *Nat Rev Cardiol*. 2011;8:292-300
378 28. Kearns NA, Genga RM, Enuameh MS, Garber M, Wolfe SA, Maehr R. Cas9
379 effector-mediated regulation of transcription and differentiation in human
380 pluripotent stem cells. *Development*. 2014;141:219-223
381 29. Xie X, Yang X, Wu J, Ma J, Wei W, Fei X, Wang M. Trib1 contributes to
382 recovery from ischemia/reperfusion-induced acute kidney injury by regulating
383 the polarization of renal macrophages. *Frontiers in immunology*. 2020;11:473
384 30. Yang Y, Li T, Vu TH, Ulaner GA, Hu JF, Hoffman AR. The histone code
385 regulating expression of the imprinted mouse igf2r gene. *Endocrinology*.
386 2003;144:5658-5670

387 31. Latos PA, Pauler FM, Koerner MV, Senergin HB, Hudson QJ, Stocsits RR,
388 Allhoff W, Stricker SH, Klement RM, Warczok KE, Aumayr K, Pasierbek P,
389 Barlow DP. Airn transcriptional overlap, but not its lncrna products, induces
390 imprinted igf2r silencing. *Science*. 2012;338:1469-1472
391 32. Szentirmay MN, Yang HX, Pawar SA, Vinson C, Sawadogo M. The igf2
392 receptor is a usf2-specific target in nontumorigenic mammary epithelial cells
393 but not in breast cancer cells. *J Biol Chem*. 2003;278:37231-37240
394 33. Zhang Y, Zhang Y, Xiao L, Yu TX, Li JZ, Rao JN, Turner DJ, Gorospe M,
395 Wang JY. Cooperative repression of insulin-like growth factor type 2 receptor
396 translation by microrna 195 and rna-binding protein cugbp1. *Mol Cell Biol*.
397 2017;37
398 34. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan
399 C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in
400 igf2r is associated with fetal overgrowth after sheep embryo culture. *Nat*
401 *Genet*. 2001;27:153-154
402 35. Swirski FK, Nahrendorf M. Cardioimmunology: The immune system in
403 cardiac homeostasis and disease. *Nat Rev Immunol*. 2018;18:733-744
404 36. Vafadarnejad E, Rizzo G, Krampert L, Arampatzi P, Arias-Loza AP, Nazzal Y,
405 Rizakou A, Knochenhauer T, Bandi SR, Nugroho VA, Schulz DJJ, Roesch M,
406 Alayrac P, Vilar J, Silvestre JS, Zernecke A, Saliba AE, Cochain C. Dynamics
407 of cardiac neutrophil diversity in murine myocardial infarction. *Circ Res*.
408 2020;127:e232-e249
409 37. Puhl SL, Steffens S. Neutrophils in post-myocardial infarction inflammation:
410 Damage vs. Resolution? *Frontiers in cardiovascular medicine*. 2019;6:25
411 38. Marinkovic G, Koenis DS, de Camp L, Jablonowski R, Graber N, de Waard V,
412 de Vries CJ, Goncalves I, Nilsson J, Jovinge S, Schiopu A. S100a9 links
413 inflammation and repair in myocardial infarction. *Circ Res*. 2020;127:664-676
414 39. Lee SY, Lee CM, Ma B, Kamle S, Elias JA, Zhou Y, Lee CG. Targeting
415 chitinase 1 and chitinase 3-like 1 as novel therapeutic strategy of pulmonary
416 fibrosis. *Frontiers in pharmacology*. 2022;13:826471
417 40. Kruzel ML, Zimecki M, Actor JK. Lactoferrin in a context of inflammation-
418 induced pathology. *Frontiers in immunology*. 2017;8
419 41. Frodermann V, Nahrendorf M. Neutrophil–macrophage cross-talk in acute
420 myocardial infarction. *European Heart Journal*. 2016;38:198-200

421 42. Othman A, Sekheri M, Filep JG. Roles of neutrophil granule proteins in
422 orchestrating inflammation and immunity. *FEBS J*. 2022;289:3932-3953
423 43. Pringle KG, Kind KL, Thompson JG, Roberts CT. Complex interactions
424 between hypoxia inducible factors, insulin-like growth factor-ii and oxygen in
425 early murine trophoblasts. *Placenta*. 2007;28:1147-1157
426 44. Kang JX, Bell J, Leaf A, Beard RL, Chandraratna RA. Retinoic acid alters the
427 intracellular trafficking of the mannose-6-phosphate/insulin-like growth factor
428 ii receptor and lysosomal enzymes. *Proc Natl Acad Sci U S A*. 1998;95:13687-
429 13691
430 45. Chavez M, Chen X, Finn PB, Qi LS. Advances in crispr therapeutics. *Nature*
431 *reviews. Nephrology*. 2023;19:9-22
432 46. Musunuru K. Crispr and cardiovascular diseases. *Cardiovasc Res*.
433 2023;119:79-93
434

435 **FIGURE LEGENDS**

436 **Fig.1 IGF2R expression in ischemic or reperfused hearts**. (**A**) Immunoblotting of
437 IGF2R and GAPDH (loading control) in mice after 45min myocardial ischemia (MI)
438 induced by left anterior descending artery ligation or releasing ligature (I/R, ischemia-
439 reperfusion) for 1 to 6 hours. (**B**) Immunostaining of IGF2R and cTnT in the ischemic
440 heart reperfused for 1 hour. Scale bar, 50μm. Arrow indicates noncardiac cells. (**C**)
441 Single-cell profiling of IGF2R expression in normal mouse heart (reanalysis of
442 GSM2967050). Cell phenotype annotation: 1-cardiac muscle cell; 2-fibroblast; 3-
443 endothelial cell; 4-Endocardial cell; 5-cardiac neuron; 6-leukocyte; 7-myofibroblast;
444 8-smooth muscle cell. (**D**) Schematic of transgenic mouse models for conditional
445 knockout (cKO) of myocardial IGF2R. (**E**) Expression of IGF2R mRNA in the Ctrl
446 ($Igf2r^{fl/fl}$) or cKO ($Myh6^{MerCreMer};Igf2r^{fl/fl}$) hearts after the tamoxifen (TAM) diet for 4
447 weeks. (**F**) Expression of IGF2R protein in the Ctrl or cKO hearts after the TAM diet
448 for 4 weeks.

449 **Fig.2 IGF2R-cKO enhances cardiac contractile functions**. (**A**) Schematic of the
450 warm I/R model. Myocardial ischemia (MI) induced by left anterior descending artery
451 (LAD) ligation for 45min and reperfusion by releasing the ligature. (**B**) Heart function
452 of control and cKO mice assessed by echocardiography in 1 week after the warm I/R.
453 (**C-D**) Heart function of control and cKO mice assessed by echocardiography in 4
454 weeks after the warm I/R. EF-ejection fraction; FS-fractional shortening; IVSd-

455 interventricular septal end-diastole; IVSs-interventricular septal end-systole. (**E**)
456 Schematic of the cold I/R model. Collecting and perfusing donor hearts with 4°C HTK
457 solution and keeping on ice until transplant into the abdomen of syngeneic recipients.
458 (**F**) The rebeating time (after anastomosis) for ventricular contraction restoration of
459 control or cKO heart grafts (preserved for 6, 12, or 24 hours in cold HTK). (**G**)
460 Survival curves of control or cKO heart grafts after transplant. The complete cessation
461 of a heartbeat is the endpoint. (**H-I**) Heart rate, pressure difference (PD), left ventricle
462 systolic pressure (LVSP), left ventricle end-diastolic pressure (LVEDP), maximum
463 dp/dt in the systolic period, and maximum dp/dt in the diastolic period of control or
464 cKO heart grafts (preserved for 12 hours in HTK) on day 1 post-transplant. (**J**) The
465 serum creatine kinase (CK) or lactate dehydrogenase (LDH) levels of control or cKO
466 heart grafts (preserved for 12 hours in HTK) on day 1 post-transplant.
467 **Fig.3 Transcriptional signatures changed by IGF2R-cKO**. (**A**) Principal
468 component (PC) analysis of the global gene profiling in the control or cKO heart at
469 normal or I/R (12-hour cold HTK and transplant reperfusion for 12 hours) conditions.
470 (**B-C**) Heatmap and hierarchical clustering analysis of differentially expressed genes
471 in cKO heart as compared to the control. The examples of different gene clusters are
472 shown with their functional annotations (GO). (**D**) Top enriched GO terms of
473 upregulated or downregulated genes in the cKO heart compared with the control. (**E**)
474 GSEA analysis of various GO terms related to the upregulated or downregulated
475 genes in the cKO heart compared with the control.
476 **Fig.4 IGF2R binds to granulocyte-derived inflammatory factors**. (A)
477 Representative silver staining image of immunoblotting of IgG or anti-IGF2R
478 antibody pulled-down proteins from the lysed control or cKO hearts. Red rectangles
479 labeling the bands for mass spectrometry analysis. (**B**) Venn's diagram comparing the
480 lists of genes upregulated or downregulated in cKO hearts and identified peptides or
481 proteins from the selected bands from (A). Five factors identified from the integrated
482 analysis are listed. (**C**) Single-cell profiling of Ngp, Chil3 (Chi3l3), S100a9, or Ltf
483 expression in normal mouse hearts (GSM2967050) or bone marrow (GSM2967055).
484 Cell phenotype annotation: 1-granulocytes; 2-granulocytopoietic cell; 3-monocyte.
485 (**D-E**) Representative hematoxylin and eosin staining (D) or Masson's trichrome
486 staining (E) of the control or cKO heart grafts after I/R (12-hour cold HTK and
487 transplant reperfusion for 4 weeks).

488 **Fig.5 Inhibition of IGF2R expression reduces cell death under hypoxia** (**A**)
489 Immunostaining of IGF2R in H9C2 cardiomyoblasts before or after hypoxia-
490 reoxygenation. Scale bar, 50μm. (**B**) Immunoblotting of IGF2R in total cell proteins
491 from H9C2 myoblasts treated with or without hypoxia-reoxygenation. (**C**)
492 Immunostaining of IGF2R with co-staining of wheat germ agglutinin (WGA) in
493 H9C2 cells before or after hypoxia. Arrowhead indicates the cell plasma membrane.
494 Scale bar, 50μm. (**D**) Immunoblotting of IGF2R after subcellular (membrane or
495 cytosolic) fractions from H9C2 cells before or after hypoxia and reoxygenation. (**E**):
496 H9C2 myoblasts were transduced with the doxycycline (Dox) inducible CRISPRi
497 targeting IGF2R's promoter region. P-Tet-ON: Pomoter with tetracycline-inducible
498 activation element. (**F**): Immunoblotting of IGF2R and caspase3 in H9C2 cells
499 exposure to hypoxia/reoxygenation.
500

Figure 1

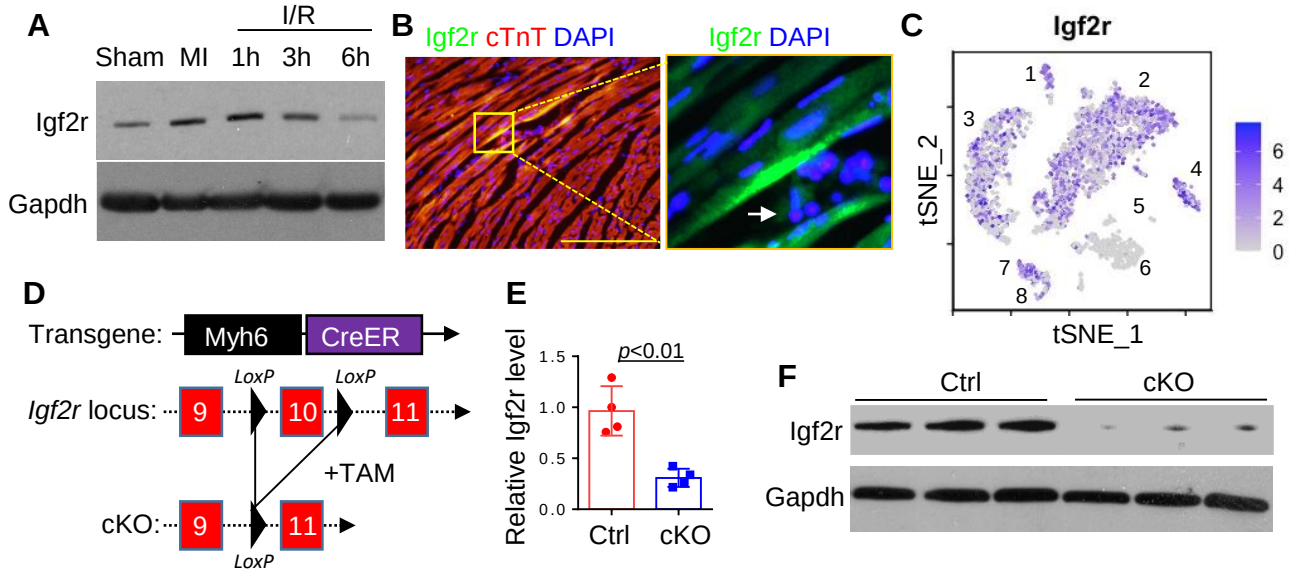

A
I/R
Sham MI 1h 3h 6h
Igf2r
Gapdh
B
Igf2r cTnT DAPI
Igf2r DAPI
C
Igf2r
tSNE_2
tSNE_1
1
2
3
4
5
6
7
8
6
4
2
0
D
Transgene:
Myh6
CreER
Igf2r locus:
LoxP
LoxP
9
10
11
+TAM
cKO:
9
11
LoxP
E
Relative Igf2r level
p<0.01
1.5
1.0
0.5
0.0
Ctrl
cKO
F
Ctrl
cKO
Igf2r
Gapdh

Figure 2

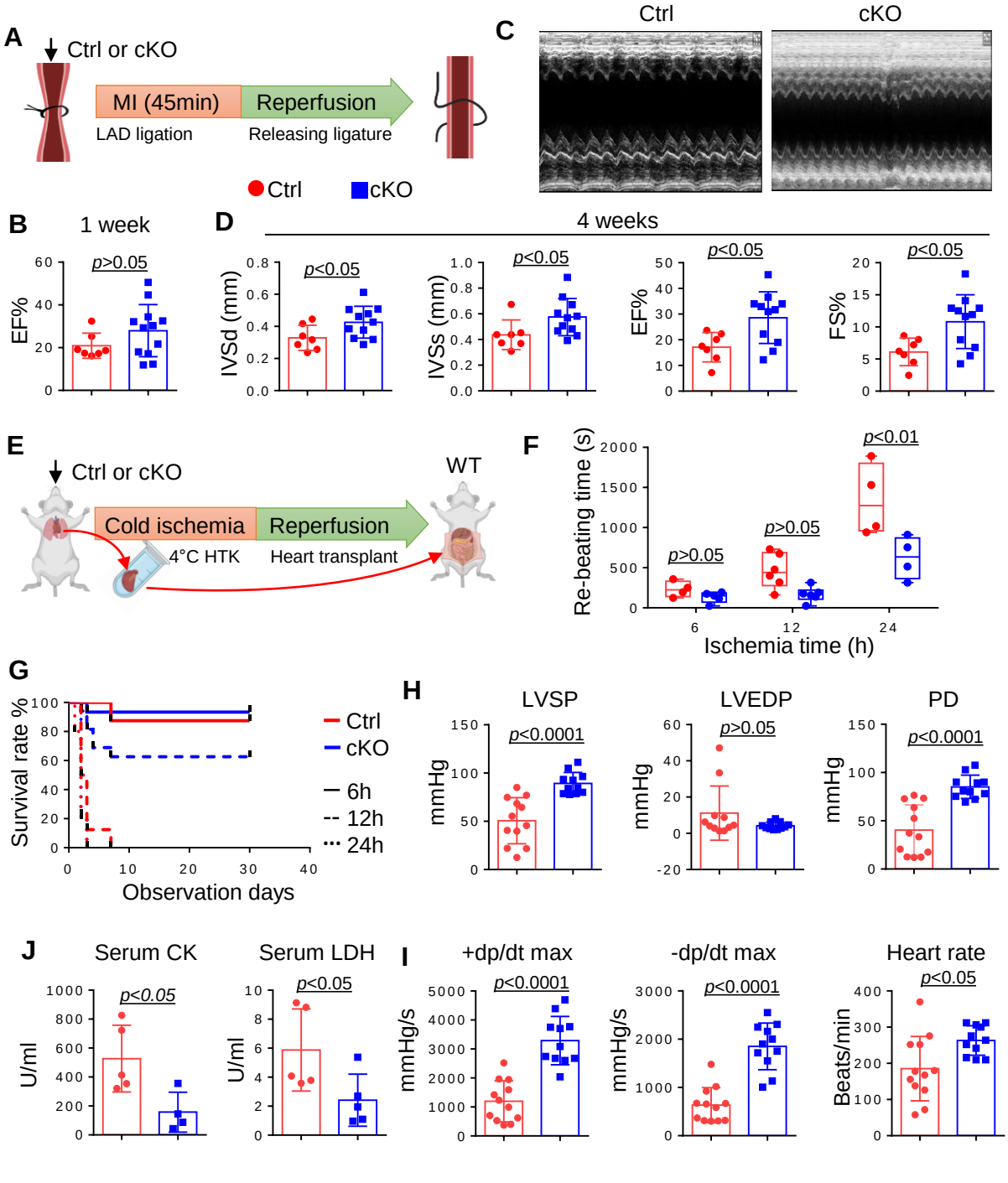
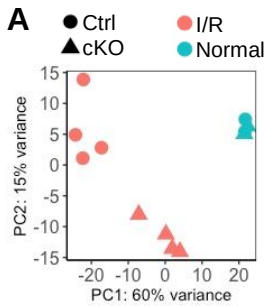

A
Ctrl or cKO
MI (45min)
LAD ligation
Reperfusion
Releasing ligature
Ctrl
cKO
C
Ctrl
cKO
B
1 week
EF%
p>0.05
D
4 weeks
IVSd (mm)
p<0.05
IVSs (mm)
p<0.05
EF%
p<0.05
FS%
p<0.05
E
Ctrl or cKO
Cold ischemia
4°C HTK
Reperfusion
Heart transplant
WT
F
Re-beating time (s)
p>0.05
p>0.05
p<0.01
Ischemia time (h)
G
Survival rate %
Observation days
Ctrl
cKO
6h
12h
24h
H
LVSP
p<0.0001
LVEDP
p>0.05
PD
p<0.0001
mmHg
J
Serum CK
p<0.05
Serum LDH
p<0.05
U/ml
I
+dp/dt max
p<0.0001
-dp/dt max
p<0.0001
Heart rate
p<0.05
mmHg/s
Beats/min

Figure 3



C

GO of cluster-a:

DNA repair (Rpain, Fen1, Prkdc, Sirt6, Lig4, etc.)
Cell cycle (Rb1, Rabgap1, Cdkn1, Cdk5, etc.)
Negative regulation of cell death (Ucn, Fgf2, etc.)
Autophagy (Tfeb, Atg13, Ulk3, Atp13a2, etc.)

GO of cluster-b:

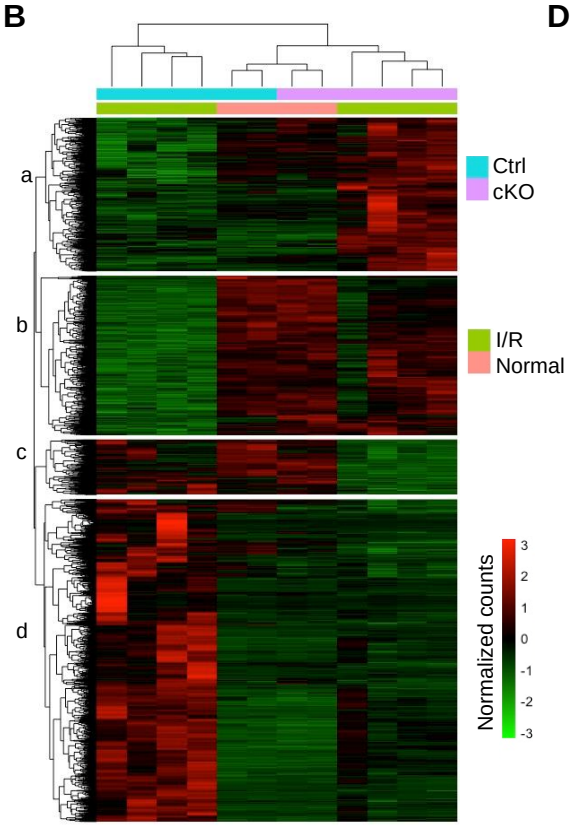
Cell division (Dca3, Aurka, Septin10, Nek1, etc.)
Cilium assembly (Ift140, Fbf1, Ift80, Wdr35, etc.)
Autophagosome assembly (Ift20, Nprl3, etc.)
Fatty acid metabolic process (Lypla1, Cpt1a, etc.)

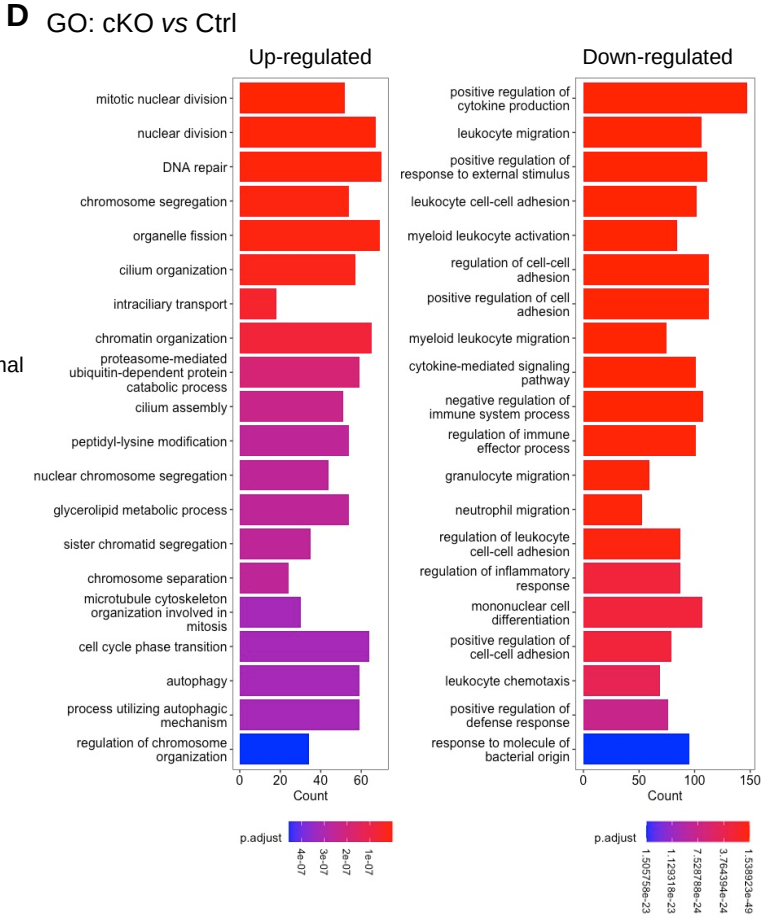
GO of cluster-c:

Inflammatory response (Csf1r, Ptgir, Ly86, etc.)
Apoptotic mitochondrial changes (Ppif, Hk2, etc.)
Cellular response to hypoxia (Egln3, Fndc1, etc.)
Angiogenesis (Dll4, Nrp2, Esm1, Vegfa, Apln , etc.)

GO of cluster-d:

Innate immune response (Cd84, Il23a, Tlr1, etc.)
Neutrophil chemotaxis (Ccl11, Cxcl1, S100a8, etc.)
Apoptotic process (Bcl2l1, Casp8, Nfkb1, etc.)
IL1β production (Nlrp3, Casp1, Casp4, Aim2, etc.)





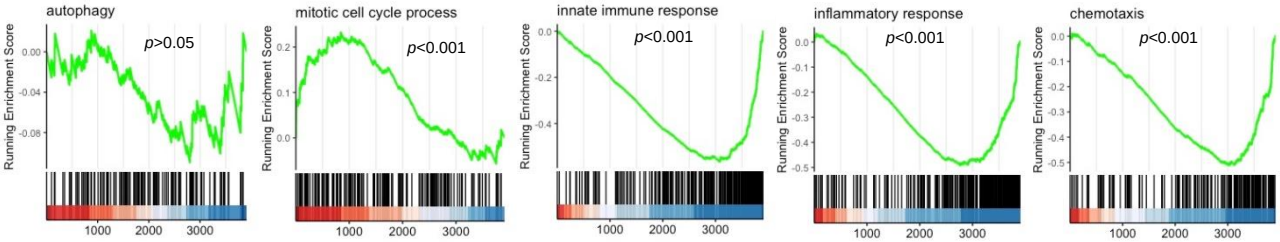


Figure 4

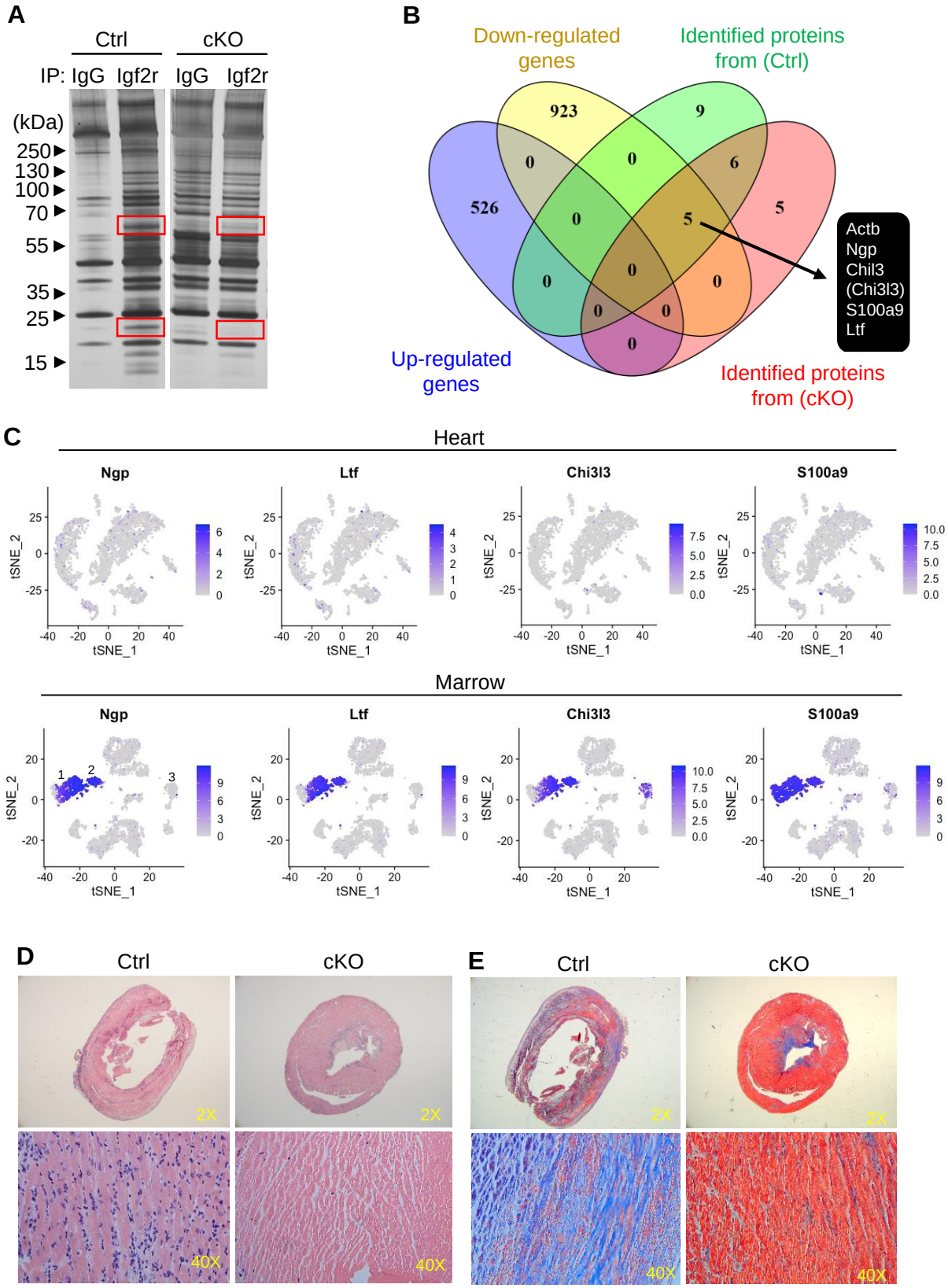
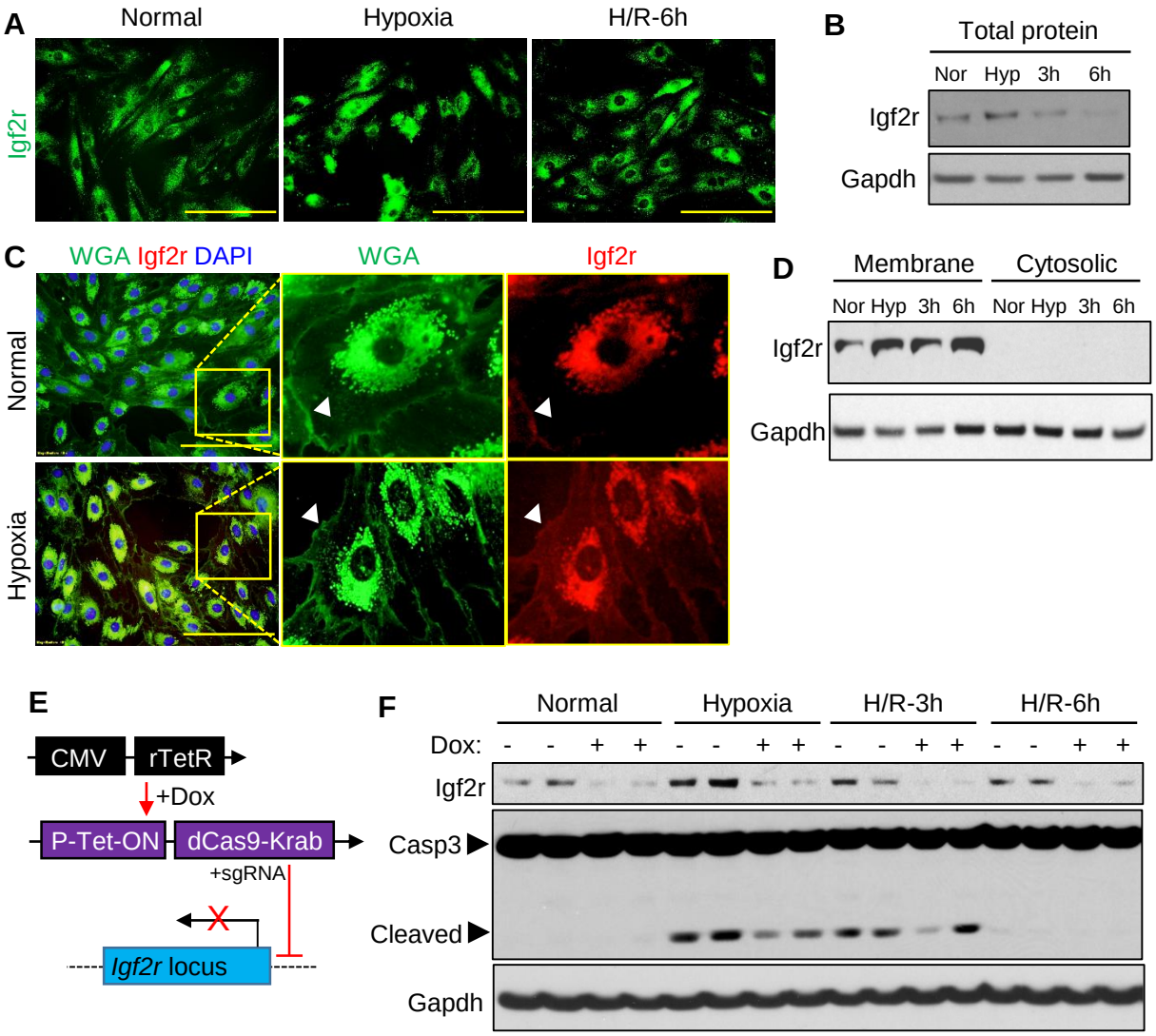

A
Ctrl
cKO
IP: IgG Igf2r IgG Igf2r
(kDa)
250
130
100
70
55
35
25
15
B
Down-regulated genes
Identified proteins from (Ctrl)
Up-regulated genes
Identified proteins from (cKO)
923
9
526
5
0
6
Actb
Ngp
Chil3 (Chi3l3)
S100a9
Ltf
C
Heart
Ngp
Ltf
Chi3l3
S100a9
tSNE_1
tSNE_2
Marrow
D
Ctrl
cKO
2X
40X
E
Ctrl
cKO
2X
40X

Figure 6


A
Normal
Hypoxia
H/R-6h
Igf2r
B
Total protein
Nor Hyp 3h 6h
Igf2r
Gapdh
C
WGA Igf2r DAPI
WGA
Igf2r
Normal
Hypoxia
D
Membrane
Cytosolic
Nor Hyp 3h 6h Nor Hyp 3h 6h
Igf2r
Gapdh
E
CMV
rTetR
+Dox
P-Tet-ON
dCas9-Krab
+sgRNA
Igf2r locus
F
Normal
Hypoxia
H/R-3h
H/R-6h
Dox: - - + + - - + + - - + + - - + +
Igf2r
Casp3
Cleaved
Gapdh