

1 **Invigorating human MSCs for transplantation therapy via Nrf2/DKK1**
2 **co-stimulation in a mice acute-on-chronic liver failure model**

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19

20 **ABSTRACT**

21 Boosting stem cell resilience against an extrinsically harsh recipient environment is critical
22 to therapeutic efficiency of stem cell-based transplantation innovations in liver disease
23 contexts. We aimed to establish the efficacy of a transient plasmid-based preconditioning
24 strategy to boost mesenchymal stromal cells (MSCs) capacity for
25 anti-inflammation/antioxidant defense and paracrine actions on recipient hepatocytes. In
26 MSCs, the master antioxidant regulator Nrf2 was found to bind directly to the antioxidant
27 response element in the DKK1 promoter region. Activation of Nrf2 and DKK1 enhanced
28 the anti-stress capacities of MSCs *in vitro*. In an acute-on-chronic liver failure (ACLF)
29 murine model, transient co-overexpression of Nrf2 and DKK1 via plasmid transfection
30 markedly improved MSC resilience against inflammatory and oxidative assaults, boosted
31 MSC transplantation efficacy and promoted recipient liver regeneration because of a shift
32 from the activation of the anti-regenerative IFN- γ /STAT1 pathway to the pro-regenerative
33 IL-6/STAT3 pathway in the liver. Moreover, specific ablation of DKK1 receptor CKAP4 but
34 not LRP6 in recipient hepatocytes nullified therapeutic benefits from MSC transplantation.
35 In long-term observations, tumorigenicity was undetected in mice following transplantation
36 of such transiently preconditioned MSCs. In conclusion, co-stimulation of Nrf2/DKK1
37 signaling decisively and safely improves the efficacy of human MSC-based therapies in
38 mouse ACLF models through apparently CKAP4-dependent paracrine mechanisms.

39 **KEYWORDS:** Mesenchymal stromal cells; ACLF; Nrf2/DKK1; CKAP4; oxidative stress

40

41 INTRODUCTION

42 As a vital organ governing organismal homeostasis, the liver is continually exposed to an
43 extraordinary array of biological/chemical toxins, some of which bear profound
44 implications for severe liver diseases. To illustrate, bacterial endotoxins (e.g.
45 lipopolysaccharide, LPS) arising from dysregulated gut microbiota are known to drive
46 severe hepatitis or liver failure through the portal vein (Wiest et al., 2017).
47 Acute-on-chronic liver failure (ACLF) is a clinical syndrome defined by liver failure with
48 pre-existing chronic liver injury. It is characterized by an acute liver insult and a rapid
49 deterioration of liver functions and with high short-term mortality (Arroyo et al., 2016;
50 Hernaez et al., 2017). The main etiologies for the acute liver insult include alcohol drinking,
51 viral hepatitis (e.g. HBV), and drug-induced liver injury (DILI), while the most frequently
52 documented etiologies for the pre-existing chronic liver injury of ACLF include chronic
53 alcoholic consumption and HBV infection (Gustot and Jalan, 2019; Sarin and Choudhury,
54 2016; Zaccherini et al., 2021). Unfortunately, there is no specific effective treatment
55 available for ACLF patients and currently treatment is based on organ support and
56 complication resolution. Since the prognosis of ACLF probably depends on the control of
57 bacterial infection and the recovery of multi-organ injury, early identification and treatment
58 of the precipitating factors (e.g. bacterial infections, gastrointestinal bleeding, alcoholism,
59 drug toxicity, and HBV reactivation) are essential (Hernaez *et al.*, 2017; Sarin et al., 2019).
60 Liver transplantation is likely the only curative treatment for ACLF patients with organ
61 failure development in the presence of cirrhosis, especially extrahepatic organ failures
62 (Trebicka et al., 2020). However, due to the lack of donor organs, liver transplantation is
63 often considered to be contraindicated when the survival rate after transplantation is lower
64 than that without transplantation (Cullaro et al., 2020). In this context, an artificial liver
65 assist device would be desirable for spontaneous liver regeneration support and proper
66 liver transplantation preparation. Current data showed that only therapeutic plasma
67 exchange could improve the survival of patients with acute liver failure (and possibly
68 ACLF). Molecular adsorbent recirculating system failed to show improvement in survival in
69 patients with ALF or ACLF (Larsen, 2019). Therefore, the development of sustainable,

70 cost-effective complementary treatment modalities for ACLF is urgently needed.

71 In recent years, exploration of human mesenchymal stromal cell (MSC)
72 transplantation has been incentivized to treat ALF and ACLF in both experimental models
73 and clinical patients (Kong et al., 2020; Liang et al., 2018; Shi et al., 2017; Shi et al., 2012).
74 While still in its formative stage, MSC-based treatments have yielded generally
75 encouraging results wherein MSCs improved recipient liver conditions by boosting hepatic
76 regenerative capacity and exerting immuno-regulatory effects via paracrine actions on
77 local hepatocytes (Wang et al., 2021; Yuan et al., 2019). A major challenge in achieving
78 efficacious MSC-based therapies in the clinic lies in the poor survival rates of stem cells
79 after transplantation (Burst et al., 2010). This problem is at least partially attributable to a
80 pro-inflammatory and pro-oxidant host environment prevailing at the injured sites. Indeed,
81 enhancement of endogenous antioxidant capacities of transplanted MCSs has been
82 variously proposed to improve transplantation efficacy (Dernbach et al., 2004; Drowley et
83 al., 2010; Zeng et al., 2015), though clinically relevant strategies remain to be established.
84 For example, several small-molecule antioxidants or ROS (reactive oxygen species)
85 scavengers including edaravone and N-acetyl-L-cysteine (NAC) have been explored to
86 enhance stem cell anti-stress responses, but there has not been definite evidence that
87 these compounds can sustainably boost MSC resilience due to a paucity of mechanistic
88 details. Since Nrf2 (nuclear factor erythropoietin-derived 2-like 2) displays protective
89 impact on stem cell biology in response to various environmental cues, via the regulation
90 of pluripotency factors, redox homeostasis, aging, and cellular stress responses (Dai et al.,
91 2020), activation of Nrf2 seems to be a plausible method for the enhancement of efficacy
92 of stem cell transplantation (Malik et al., 2013). Unfortunately, the underlying mechanisms
93 and subsequent profile of SC secretory factors are poorly understood. In addition, a lack
94 of information on the direct molecular targets of transplanted MSCs' paracrine actions also
95 impedes advances in therapeutic inventions. While several kinds of such mechanisms
96 have been brought to light in animal disease models, the key potential targets mediating
97 MCS benefits call for rigorous scrutiny in molecular and biochemical approaches (Kusuma
98 et al., 2017; Tachibana et al., 2017).

99 Inspired by these questions, we here endeavored to demonstrate that Nrf2 directly
100 boosted cellular antioxidant responses and confer hepatoprotectant capabilities in part
101 through the regulation of DKK1 secretion from human MSCs. Collectively, coordinated
102 stimulation of the Nrf2/DKK1 signaling contributed to an enhanced anti-stress capacity of
103 MSCs. Gratifyingly, activation of Nrf2 and DKK1 signaling via transient plasmid
104 preconditioning of transplanted MSCs effectively and safely boosted the transplantation
105 efficacy *in vivo* in a novel murine model of ACLF, partly due to accelerated liver
106 regeneration because of a shift from the activation of the anti-regenerative IFN- γ /STAT1
107 pathway to the pro-regenerative IL-6/STAT3 pathway. In a paracrine manner, secreted
108 DKK1 from transplanted MSC exerted pro-resolving and reparative effects on recipient
109 mouse hepatocytes via the DKK1 receptor, cell surface cytoskeleton-associated protein 4
110 (CKAP4).

111

112 **RESULTS**

113 Nrf2 promotes the anti-stress capacity of MSC via direct regulation of DKK1

114 Since the transplantation of MSC preconditioned with the minocycline or doxycycline
115 protects against ischemic injury in murine models via Nrf2 activation (Malik *et al.*, 2013;
116 Sakata *et al.*, 2012), we first examined whether treatment of TNF- α /H₂O₂ (tumor necrosis
117 factor-alpha plus hydrogen peroxide), a well-characterized reactive oxygen
118 intermediates- and inflammation inducer-challenged ACLF-like cell model (Bátkai *et al.*,
119 2007; Gilston *et al.*, 2001; Kudo *et al.*, 2009), altered the basal Nrf2 activity and the
120 release of key soluble cytokines/chemokines. A 24-h treatment with TNF- α /H₂O₂
121 significantly promoted the activity of Nrf2 in MSCs, as well as the translational and
122 secreted levels of Wnt canonical pathway inhibitor Dickkopf-1 (DKK1) (Figure 1A).
123 TNF- α /H₂O₂ treatment also enhanced the secretion of pro-inflammatory
124 cytokines/chemokines (IL-1 β , IL-6, MCP-1, and RANTES) and anti-inflammatory cytokine
125 (IL-10) from MSCs, indicating an ACLF-comparable inflammatory environment in the cell
126 culture system (Figure supplement 2). Next, we sought to determine whether the effects

127 observed were because of a direct association between Nrf2 and *Dkk1* gene promoter. By
128 using bioinformatics analysis, we found the presence of antioxidant response element
129 sequences (AREs) at position -96 from the *Dkk1* transcription start site, which had
130 similarity to the ARE consensus sequence observed in other Nrf2 target genes (e.g.
131 NQO1, HMOX1, and SOD1). In consistent with the result, Nrf2-containing plasmid
132 significantly reduced the luciferase activity of DKK1-bearing plasmid, which was
133 hampered when DKK1 was mutated from TGACTCTGC to ATCGAGATA (Figure 1B).
134 Moreover, a dose-dependent increase in *Dkk1* promoter activity was seen when *Nrf2* was
135 knocked-down by siRNA (Figure 1C). Then the basal expression of *Nrf2* and *Dkk1* was
136 overexpressed or inhibited by transfection with gene ORF (open reading frame)-bearing
137 plasmid or shRNA, respectively, at the time of 48-h before the treatment of TNF- α /H₂O₂
138 (Figure 1D). Silencing of Nrf2 reduced cell viability in TNF- α /H₂O₂-challenged MSCs,
139 whereas overexpression of Nrf2 or DKK1 evidently improved cell viability. Inhibition of
140 DKK1 did not influence the cell viability after TNF- α /H₂O₂ challenge (Figure 1E). We
141 observed consistent changes of MSCs apoptotic ratio after the manipulations of Nrf2 and
142 DKK1 basal expression (Figure 1E). In addition, protein expression changes of the cell
143 cycle regulator PCNA (proliferating cell nuclear antigen) and apoptotic negative regulator
144 Bcl-2 reflected influences of Nrf2/DKK1 signaling in MSCs viability and apoptosis,
145 respectively (Figure 1F). This result was further strengthened by the activity changes of
146 caspase-3/8 of MSCs (Figure 1G). Since endogenous production of excessive cellular
147 and mitochondrial reactive oxygen species (ROS) in MSCs can arise as a direct
148 consequence of extrinsically imposed TNF- α /H₂O₂ toxicity (Gilston *et al.*, 2001), we
149 ventured to verify the effects of Nrf2/DKK1 modulation on oxidant-induced cellular and
150 mitochondrial dysfunction by detection of oxidative events with CellRox (cellular oxidative
151 stress probe) and MitoSOX (mitochondrial superoxide probe), respectively. As anticipated,
152 exposure of TNF- α /H₂O₂ elevated endogenous production of cellular/mitochondrial ROS
153 of MSCs, which was alleviated by Nrf2 or DKK1 overexpression. Knockdown of Nrf2 or
154 DKK1 slightly exacerbated cellular/mitochondrial ROS production of MSCs (Figure 1H
155 and 1I). Collectively, Nrf2 promotes the anti-stress capacity of MSC via direct regulation of
156 DKK1.

157

158 Mobilization of the Nrf2/DKK1 signaling in MSC led to a moderation of
159 cellular/mitochondrial ROS production

160 In order to substantiate mechanistically how Nrf2/DKK1 signaling contributes to MSCs
161 resilience against TNF- α /H₂O₂-induced stress, we validated the interrelations between
162 this pathway and ROS production in human MSCs by using MitoQ (mitochondrial ROS
163 scavenger) and N-acetylcysteine (NAC; total cellular antioxidant) in the presence of
164 TNF- α /H₂O₂. Consistent with our assumptions, co-treatment with MitoQ or NAC
165 significantly ameliorated TNF- α /H₂O₂-induced cell damages in several aspects, including
166 an increase in cell viability/PCNA expression and a reduction in apoptosis and
167 cellular/mitochondrial ROS production (Figure 2A-2C). Nrf2 activity of MSCs was
168 increased by TNF- α /H₂O₂ exposure, but was re-balanced by co-treatment with MitoQ or
169 NAC. Similarly, TNF- α /H₂O₂ challenge evidently reduced p38 MAPK phosphorylation and
170 DKK1 protein expression, which were substantially restored by MitoQ/NAC co-treatment
171 (Figure 2D). It is noteworthy that the decreased ERK phosphorylation seen during
172 TNF- α /H₂O₂ exposure was further suppressed by MitoQ/NAC (Figure 2D). Importantly,
173 previous studies have postulated cross-regulation between MAPKs and DKK1 in cancer
174 cells and T cells (Browne et al., 2016; Chae et al., 2016; Rachner et al., 2015), but it
175 remains largely unknown whether potential crosstalk exists between the MAPK and
176 Nrf2/DKK1 pathways in human MSCs. In our *in vitro* study on TNF- α /H₂O₂-induced cell
177 damages, we found that the provoked Nrf2 activity were further enhanced by SB203580
178 (p38 MAPK inhibitor) or UO126 (MEK1/2 inhibitor). Change of cellular and secreted DKK1
179 levels was in opposite to that of Nrf2, further confirmed the negative regulatory loop
180 between Nrf2 and DKK1. When Nrf2 was overexpressed by plasmid transfection, both
181 basal and TNF- α /H₂O₂-suppressed phosphorylated p38 MAPK levels were markedly
182 elevated. Contrarily, Nrf2 overexpression strongly suppressed ERK phosphorylation
183 under basal and TNF- α /H₂O₂-treated conditions (Figure 2E). Overexpression of DKK1 in
184 MSCs visibly increased phosphorylated p38 MAPK levels but decreased phosphorylated
185 ERK levels with or without TNF- α /H₂O₂ exposure (Figure 2E). Moreover, TNF- α /H₂O₂

186 exposure diminished the cellular levels of NAD(P)H dehydrogenase [quinone] 1 (NQO-1)
187 and heme oxygenase-1 (HO-1) (Figure supplement 3A), which are Nrf2-regulated
188 antioxidant enzymes important to stem cell homeostasis (Chen et al., 2014).
189 Pharmacological inhibition of p38 MAPK and ERK further reduced and restored their
190 expression, respectively, while Nrf2 overexpression significantly improved both the basal
191 and TNF- α /H₂O₂-repressed levels of NQO-1 and HO-1 (Figure supplement 3A). When
192 endogenous expression of NQO-1 or HO-1 in either type of human MSCs was silenced by
193 specific shRNAs, the ameliorative effects of Nrf2 or DKK1 overexpression on cell injury
194 were drastically curtailed (Figure supplement 3B-3C), suggesting a dependence on
195 NQO-1 and HO-1 as distal mediators in MSC anti-stress response. Collectively,
196 mobilization of the Nrf2/DKK1 signaling in MSC led to a moderation of
197 cellular/mitochondrial ROS production, partly via the antioxidant actions of NQO-1 and
198 HO-1.

199

200 Transient co-overexpression of Nrf2 and DKK1 boosts MSC resistance against stress

201 Although Nrf2 directly and negatively regulates DKK1 expression, and *vice versa*, we
202 found overexpression of each of them could alleviated inflammation- and oxidative
203 stress-induced cell injury in MSCs. To maximize the alleviative effects and to avoid the
204 negative regulating loop between them, we attempted to co-overexpress Nrf2 and DKK1
205 by constructing an optimized pIRES2-Nrf2-DKK1 expression plasmid (Figure 3A) and
206 transfected MSCs with it for 5 days (approximate the same duration where MSCs need to
207 endure the harsh effects of local stress following transplantation) to verify its protein
208 inductive potential. Following transfection, protein expression of Nrf2, total DKK1 and
209 secreted DKK1 in human MSCs began to rise at day 2, peaked at day 3 and returned to
210 near-basal levels at day 5 post-transfection (Figure 3B). Importantly, transient transfection
211 of this plasmid did not alter MSC viability or MSC differentiation potential (Figure
212 supplement 4). Based on this observation, we subjected MSCs to added TNF- α /H₂O₂
213 challenge at day 3 post-transfection for another 24 h to evaluate the cells' anti-stress
214 capacity gained from plasmid transfection. As anticipated, pIRES2-Nrf2-DKK1 plasmid

215 transfection significantly restored cell viability, reduced apoptosis, and dampened
216 cellular/mitochondrial ROS production in MSCs without altering their basal status (Figure
217 3C-3E).

218

219 Co-stimulation of Nrf2 and DKK1 expression improves MSC transplantation efficacy in an
220 ACLF mice model

221 By using a novel ACLF mice model combining chronic liver injury, acute hepatic insult, and
222 bacterial infection that phenocopies some of the key clinical features of ACLF patients
223 (Xiang et al., 2020), we tested the MSC-protective effects of Nrf2/DKK1 co-stimulation
224 strategy (Figure 4A). Empirically, both death counts of ACLF mice (during a 9-d time
225 window) were evidently mitigated by the transplantation of human MSCs. In particular,
226 preconditioning MSCs with the pIRES2-Nrf2-DKK1 plasmid prior to transplantation further
227 enhanced the protective effects in either type of mice. No further mouse death was seen
228 in all groups during an extended 9-day observation period (Figure 4B). High levels of
229 serum ALT and total bilirubin (TBIL), elevation of circulating neutrophils and blood urea
230 nitrogen (BUN), and reduction of renal microvascular flow were all significantly alleviated
231 by MSCs transplantation, and further strengthened by pIRES2-Nrf2-DKK1 plasmid
232 preconditioning (72-h post *K.P.* administration data presented here; Figure 4C and 4D).
233 ACLF-induced severe pathological liver damages including necrosis, inflammation,
234 fibrosis, and lipid peroxidation were also ameliorated by MSCs and plasmid
235 preconditioning (Figure 4E). Successfully homed human MSCs in the damaged mice liver
236 were labelled with human nuclear antigen (hNA) staining, DKK1 protein, and alpha-1
237 antitrypsin (α AT) protein which showed that transfection with the pIRES2-Nrf2-DKK1
238 plasmid significantly improved the homing efficacy of MSCs, which was closely associated
239 with the accelerated liver regeneration, as demonstrated by hepatic Ki-67 staining (Figure
240 4F). Since this ACLF model was reported to induced evident liver fibrosis, we also
241 investigated the possible fibrotic amelioration after MSCs transplantation.

242

243 MSCs preconditioned with pIRES2-Nrf2-DKK1 resolves ACLF injury by enhancing the
244 pro-regenerative IL-6/STAT3 pathway but attenuating the anti-regenerative IFN- γ /STAT1
245 pathway

246 To investigate the mechanisms for MSCs transplantation-induced liver regeneration and
247 fibrosis resolution, we measured serum cytokines and found that in ACLF mice, IL-6
248 protein level was significantly inhibited while IFN- γ protein level was significantly provoked
249 when compared with that of control mice. In the liver, the mRNA level changes of *Ilf6* and
250 *Bcl2* corresponded with that of serum IL-6 protein, while *Ifna* and *Stat1* mRNA level
251 changes corresponded with that of serum IFN- γ protein (Figure 5A and 5B). Western blot
252 analyses revealed that ACLF mice had enhanced phosphorylated levels of STAT1 and
253 STAT3 vs. control mice, which was suppressed after the transplantation with MSCs, with
254 or without pIRES2-Nrf2-DKK1 plasmid preconditioning (Figure 5C and 5D). In contrast to
255 STAT activation, the expression of cyclin D1 was lower in mice with ACLF compared to
256 those control mice (Figure 5C and 5D).

257

258 Hepatocyte membrane receptor CKAP4 but not LRP6 mediates MSC-based recipient liver
259 repair

260 As a canonical inhibitor of Wnt signaling, DKK1 has previously been shown to be secreted
261 by MSCs to ameliorate tissue injury or reduce liver fibrosis (Prockop et al., 2003; Yang et
262 al., 2017). In a similar vein, we speculated whether DKK1 receptor(s) on hepatocyte cell
263 membranes transduces DKK1 signaling from transplanted human MSC to promote repair
264 processes in murine recipient hepatocytes. Thus, by using AAV8-ligated shRNAs, we
265 knocked down the expression two well-documented hepatocyte DKK1 receptors,
266 cytoskeleton-associated protein 4 (CKAP4) and low-density lipoprotein receptor-related
267 protein 6 (LRP6) (Kimura et al., 2016), specifically in the liver (Figure 6A). Compared with
268 their WT littermates, LRP6 conditional knockdown (CKD) mice showed a similar death
269 rate, while CKAP4 CKD mice had a greater death rate upon ACLF challenge (Figure 6B).
270 Changes in serum ALT, liver histology, and hepatic injury markers were consistent (72-h

271 post *K.P.* administration data presented here; Figure 6C and 6D; WT mice data not shown
272 here). Of note, hepatic knockdown of CKAP4 or LRP6 did not influence the hepatic hNA
273 signal, as well as human DKK1 protein and α AT protein levels, indicating that the homing
274 efficacy of MSCs transplantation, with or without pIRES2-Nrf2-DKK1 preconditioning, did
275 not rely on hepatocyte CKAP4 and LRP6 (Figure 6E). In contrast, hepatic staining of Ki-67,
276 protein expression analysis on p-Akt, cyclin D1, and PCNA demonstrated that hepatic
277 knockdown of CKAP4, when compared with LRP6 knockdown or WT mice, exhibited
278 significantly lower level of liver regeneration, which suggests that MSC-based intervention
279 for recipient liver regeneration was CKAP4-dependent (Figure 6F and 6G).

280

281 Long-term transplantation with plasmid-transfect MSCs is safe in murine models

282 Potential risks for tumorigenicity are an important safety issue in the development of
283 MSC-based therapies, particularly for viral- or plasmid-manipulated MSCs (Tolosa et al.,
284 2016). In our ACLF model, no mice developed tumor (tumor incidence rate: 0%) during
285 their long-term observation period (24 weeks). In contrast, all animals in the positive
286 control groups of healthy and ACLF models showed severe symptoms of dyspnea and
287 minimal activity from 5-6 weeks after ES-3D cell injection. Gross morphology suggests
288 that 100% of the mice developed tumors in the lungs (Table supplement 1). To assess the
289 long-term viability of donor MSCs, human albumin was measured in the mouse serum at
290 12- and 24-weeks post-transplantation. Human albumin levels were determined to be
291 about 2 times higher in the serum of ACLF mice transplanted with Nrf2/DKK1
292 preconditioned MSCs than mice with plasmid-naïve MSCs at 12-week post-injection. By
293 the 24th week, the differences in human albumin levels between mice transplanted with
294 Nrf2/DKK1 preconditioned MSCs and mice with plasmid-naïve MSCs became less
295 pronounced than in the 12th week (Figure supplement 5).

296

297 **Discussion**

298 Accumulating evidence converges on the proposition that enhancement of anti-stress

299 capacity of transplanted stem cells favorably influences therapeutic outcomes in a variety
300 of diseases, though the essential signaling pathways that regulate mechanisms therein
301 remain incompletely understood. Previous studies have shown that Nrf2 and its upstream
302 MAPK pathways are involved in antioxidant-promoted stem cell resistance against
303 exogenous stress, but their exact roles in reparative processes in liver injury require
304 further elucidation (Drowley *et al.*, 2010; Zeng *et al.*, 2015). In this context, we
305 demonstrate here that the regulatory roles of p38 MAPK and ERK in human MSC injury
306 are opposite. p38 MAPK inhibition worsened while ERK inhibition alleviated
307 TNF- α /H₂O₂-induced cell injury. Nrf2 directly bound to the ARE element of the promoter of
308 DKK1, which was shown to be indispensable for protecting transplanted MCS against
309 local stress. Although there was a negative regulation between Nrf2 and DKK1,
310 overexpression of them simultaneously exhibited the best protective effects on MSCs than
311 that of overexpression for any of them. Further analysis shows that Nrf2/DKK1
312 overexpression attenuated cellular/mitochondrial ROS production and restoration of
313 antioxidant reserves. In addition, augmented expression of Nrf2 and DKK1 increased the
314 levels of phosphorylated p38 MAPK and reduced the levels of phosphorylated ERK, to
315 form a positive feedback loop that sustains anti-stress regulation within preconditioned
316 MSCs. This finding is in agreement with several previous studies supporting a direct
317 crosstalk between MAPK and Nrf2/DKK1 in other cell types (Browne *et al.*, 2016; Kim *et*
318 *al.*, 2014; Naidu *et al.*, 2009; Niwa *et al.*, 2009).

319 Selective overexpression of key proteins to boost the anti-stress capacity of MSCs prior to
320 transplantation is a theoretically sound strategy for improving therapeutic efficacy. In
321 application contexts elsewhere, for example, forced myocardin expression in human
322 MSCs by adenoviral gene transfer promoted their cardiomyogenic differentiation and
323 transplantation efficiency in murine ischemic heart injury models (Grauss *et al.*, 2008).
324 Co-expression of the HCMV proteins US6 and US11 through retroviral vector-based
325 transfection in human MSCs successfully switched off recognition of MSCs by the
326 immune system, thus allowing a higher level of productive engraftment in the murine liver
327 after transplantation (Soland *et al.*, 2012). Nevertheless, since viral gene transfer methods

328 genetically drive host cell reprogramming via modulation of activities of target and
329 neighboring genes at the insertion site, they potentially raise safety issues of possible
330 tumor development post-transplantation, especially in clinical applications (Wang et al.,
331 2014). In comparison, transiently induced overexpression of target gene(s) in MSCs by
332 transfection with carefully conceived plasmids seem to be a relatively safe and technically
333 worthy method. Indeed, as compellingly demonstrated in this study, this alternative
334 approach can significantly ameliorate therapeutic outcomes in our clinically-relevant ACLF
335 model through the use of Nrf2/DKK1 preconditioned MSCs. Long-term observations
336 following transplantation did not suggest any adverse effects, precluding the possibility of
337 carcinogenesis.

338 In terms of mechanisms, the recipient liver signaling cascades directly molded by stem
339 cell therapies after injury have long been an enigma. It has been proposed that stem cells
340 are capable of orchestrating host hepatocyte regeneration via direct homing,
341 replenishment of functional hepatocytes, and paracrine actions (e.g. via secreting proteins
342 and extracellular vesicles for cell-to-cell communication). CKAP4 and LRP6 were reported
343 to be the direct DKK1 receptors implicated in cancer cell proliferation, with similar affinity
344 but distinct cysteine-rich domains (Kimura *et al.*, 2016). In this current study, we asked
345 which of the receptors matter and found that CKAP4, but not LRP6, mediated the
346 DKK1-induced host hepatocyte regeneration partly through Akt activation in the ACLF
347 hepatotoxicity model. Indeed, we believe that whether other DKK1 targets in hepatocytes
348 contribute to this intricate process warrants further investigation. As an important point to
349 note, however, abundant DDK-1 expression may promote hepatocellular carcinoma cell
350 migration and invasion and serve as a protein biomarker of liver cancers (Chen et al.,
351 2013; Shen et al., 2012). Thus, transient overexpression of DKK1 as an intervention
352 strategy should be viewed with caution for some patients with such cancers. Insights
353 gained on related signaling pathways underpinning the anti-stress capacity of MSCs will
354 definitively help improve the efficacy and technical maturity of MSC-based transplantation
355 therapies for other types of intractable clinical diseases.

356 In conclusion, we have herein demonstrated that the Nrf2/DKK1 signaling pathway

357 sustains and enhances MSC resilience against extrinsically imposed stress
358 post-transplantation. The MAPK member proteins p38 and ERK are also involved in this
359 process. MSC preconditioning by transiently induced co-overexpression of Nrf2 and
360 DKK1 via plasmids efficaciously and safely improved the transplantation efficacy and
361 therapeutic outcomes of human MSCs in a murine ACLF model. It is hoped that our
362 findings will help lay a theoretical foundation for further innovations of translationally
363 mature MSC-based therapies for liver diseases.

364

365 **EXPERIMENTAL PROCEDURES**

366 **MSC isolation, culture and validation of surface markers**

367 Commercially available human adipose-derived mesenchymal stromal cells (hADMSCs;
368 #HUXMD-01001) were purchased from Cyagen Biosciences (Guangzhou, China), and
369 handled according to the manufacturer's instructions. Flow cytometry was used to
370 characterize the human MSCs. For validation, the following BD Pharmingen™ monoclonal
371 antibodies (mAbs) were used: phycoerythrin (PE) conjugated mouse antibodies with
372 anti-human immunoreactivity for CD34 (#555822), CD44 (#555479), CD45 (#555483),
373 and CD105 (#560839) (BD Biosciences, San Jose, CA). Human MSCs were separately
374 incubated with the above mAbs or mouse IgG isotype control for 30 min at 4°C. Excess
375 mAbs were removed by washing twice with PBS. Cells were resuspended in 0.5 mL PBS
376 to achieve a final density of 2×10^5 cells prior to acquisition and were then analyzed by a
377 FACSCalibur flow cytometer (BD Biosciences). The surface marker expression of human
378 MSCs thus enriched was assessed by using flow cytometry following 2 passages. The
379 results indicated a high expression of CD44 and CD105 (all > 94% in both MSCs) and a
380 very low expression level of CD34 or CD45 (all < 0.5% in both MSCs) (Figure supplement
381 1).

382

383 **Plasmid construction and transfection**

384 Construction of the pIRES2-Nrf2-DKK1 plasmid, which was used to simultaneously
385 overexpress Nrf2 and DKK1 in MSCs prior to their administration, was based on a
386 pIRES2-EGFP vector (Clontech, Mountain View, CA; #6029-1). cDNA inserts from coding
387 domain sequences (CDS) of the Nrf2 (GenBank accession: NM_006164) and DKK1
388 genes (GenBank accession: NM_012242) were synthesized by PCR. EGFP gene in the
389 pIRES2-EGFP vector was then replaced by a DKK1 CDS sequence to generate a
390 pIRES2-DKK1 vector. The Nrf2 gene fragment was inserted into the pIRES2-DKK1 vector
391 at the restriction sites of SacI and BamHI. Finally, overexpression (OE) plasmids
392 containing CDS of both human Nrf2 and DKK-1 genes of Nrf2 and DKK1 were obtained by
393 PCR amplification and sub-cloning into an empty pCDNA3.1 plasmid. List of plasmid
394 constructs is detailed in Table supplement 2. Knockdown (KD) of endogenous Nrf2
395 (#NM-006164-07241504MN), DKK1 (#NM-012242-07241504MN), NQO-1
396 (#NM-000903-07241504MN), or HO-1 (#NM-002133-07241504MN) expression was
397 achieved by using corresponding human MISSION shRNAs commissioned with
398 Sigma-Aldrich (St Louis, MO). Transfection of plasmids or shRNAs into MSCs was
399 conducted by using a Lipofectamine 3000 system (#1687583; Invitrogen, Carlsbad, CA).
400 Efficiency of genetic OE or KD was verified according to the manufacturers' instructions.

401

402 **Luciferase reporter assay**

403 Approximately 1 kb upstream region of the *Dkk1* gene transcription start site (TSS)
404 containing the wild-type (WT) antioxidant response element (ARE; TGACTCTGC) or
405 mutated ARE (ATCGAGATA) was conjugated to the translation start site in the pGL3-basic
406 vector (Promega, Madison, Wisconsin). HEK-293T cells were plated in 24-well plates 24 h
407 before transfection. Briefly, HEK293T cells reached 70% confluence at the time of
408 transfection. The transfection system is as follows, 450 ng of pcDNA3.1-Nrf2 plasmid, 75
409 ng of pGL3-basic-DKK1-ARE-WT (or 75 ng of pGL3-basic-DKK1-ARE-Mut plasmid) and
410 25 ng pRL-TK were co-transfected using 1.5 µg of HG transgene reagent (IBSbio,
411 Shanghai, China). After 48 h of the transfection, cells were harvested and lysed for

412 luciferase assay. Add 20 μ l of sample and 20 μ l of Firefly Luciferase Assay Reagent to the
413 measurement tube, mix thoroughly and measure the RLU (relative light unit), while setting
414 the cell lysis buffer as a blank control well. Add 20 μ l of the prepared Renilla Luciferase
415 Assay working solution to the tested sample, and then measure the RLU after thorough
416 mixing. The RLU values detected by Firefly Luciferase are compared with those detected
417 by Renilla Luciferase, and the activation degree of the reporter gene is determined by the
418 ratio.

419

420 **Cell culture, reagents and chemicals**

421 All cell culture reagents and consumables were purchased from Gibco (Carlsbad, CA) or
422 Corning Incorporated (Corning, NY). Hydrogen peroxide (H_2O_2 ; #H1009),
423 *N*-acetyl-L-cysteine (NAC; #S0077) and methylthiazolyldiphenyl tetrazolium bromide
424 (MTT; #2128) were products from Sigma-Aldrich (St Louis, MO). Mitochondria-targeted
425 antioxidant (MitoQ) was purchased from MedChemExpress (#HY-100116; Monmouth
426 Junction, NJ). Antibodies for human proliferating cell nuclear antigen (PCNA; #ab18197),
427 Bcl-2 (#ab196495), phosphorylated p38 mitogen-activated protein kinase (MAPK) at
428 Thr180/Tyr182 (#ab4822), total p38 MAPK (#ab31828), phosphorylated p44/42 MAPK
429 (ERK) at Thr202/Tyr204 (#ab214362), total ERK (#ab17942), Dickkopf-1 (DKK1;
430 #ab93017), alpha 1 antitrypsin (α AT; #ab166610), albumin (Alb; #ab106582), Nuclear
431 factor erythroid 2-related factor 2 (NRF2; #ab92946), Cyclin D1 (#ab16663), STAT1
432 (#ab239360), phosphorylated STAT1 at S727 (#ab109461), STAT3 (#ab68153),
433 phosphorylated STAT3 at Y705 (#ab76315), Heme Oxygenase 1 (HO-1; ab189491),
434 NAD(P)H quinone dehydrogenase 1 (NQO-1; #ab80588) and
435 pan-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #ab8245) antibodies were
436 provided by Abcam (Cambridge, England). The p38 MAPK inhibitor SB203580 (#S8307)
437 and ERK inhibitor U0126 (#U120) were purchased from Sigma-Aldrich (St Louis, MO).
438 They were added (20 μ M) to the cell culture medium 2 h before toxin (e.g. LPS/ H_2O_2 or
439 ethanol) treatment. Recombinant human tumor necrosis factor-alpha was purchased from

440 PeproTech (Rocky Hill, NJ; #300-01A).

441

442 **ACLF mouse model**

443 Male 7-week-old wild-type (WT) C57BL/6J (approximately 21 g) mice were procured from
444 the Guangdong Experimental Animal Center (Guangzhou, China). The ACLF model was
445 established as previously described (Xiang *et al.*, 2020). Briefly, mice were injected
446 intraperitoneally (i.p.) with CCl₄ (0.2 ml/kg twice a week) and then an i.p. injection with
447 *Klebsiella pneumoniae* (K.P.) strain 43816 (ATCC, Manassas, VA). All experimental
448 procedures were approved by the Ethical Committee of Shenzhen Third People's Hospital
449 (SZTPH: 2016-07). For *in vivo* assessment, mouse serum and liver tissue were collected
450 at day 3 after MSC transplantation, since our previous studies showed that on sampling
451 day 3 (an experimentally optimized window for observation) would allow sufficiently
452 informative evaluation on therapeutic effects from drugs or MSCs (Liu *et al.*, 2017; Zeng *et*
453 *al.*, 2015). For *in vivo* viral injection for CKAP4/LRP6 hepatic knockdown, mice were
454 injected via the tail vein with 1×10^{12} genomic copies of AAV8 control or AAV-shRNA (5 per
455 group). Mice were maintained in a 12 h light/12 h dark cycle. After 14 days, mice were
456 fasted for 4 h at the end of the dark cycle and then sacrificed to ensure hepatic
457 downregulation of CKAP4 or LRP6, for comparison with other major organs.

458

459 **Cell viability**

460 Changes in MSC viability after specific treatment(s) were measured by using MTT assay.
461 After treatments, cells were washed by sterile phosphate buffer saline (PBS) 3 times and
462 then incubated with 5 mg/mL MTT (Sigma-Aldrich; #M2128) for 4 h, whose reaction
463 products were subsequently dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich;
464 #D2650). Absorbance of MTT was measured at 570 nm and pure DMSO was set as a
465 blank control.

466

467 **AAV8-shRNA preparation**

468 Adeno-associated virus type 8 (AAV8) was produced by transfection of AAV-293 cells with
469 three plasmids, namely: an AAV vector expressing short hairpin RNA (shRNA) targeting
470 mouse CKAP4/LRP6; an AAV helper plasmid (pAAV Helper); and an AAV Rep/Cap
471 expression plasmid. At 72 h post-transfection, cells were harvested and lysed by following
472 a freeze-thaw procedure. Viral particles were purified by means of an iodixanol
473 step-gradient ultracentrifugation method. Iodixanol was subsequently diluted, and AAV
474 was concentrated by using a 100-kDa molecular-weight cutoff ultrafiltration device. A
475 genomic titer of 2.5×10^{12} - 5×10^{12} infectious units per microliter was determined by
476 real-time quantitative PCR. To construct shRNAs, oligo-nucleotides containing sense and
477 antisense sequences were joint by a hairpin loop followed by a poly (T) termination signal.
478 The sequences targeting mouse CKAP4 (GenBank accession: NM_175451.1) or mouse
479 LRP6 (GenBank accession: NM_008514.4) as used in the experiments were 5'-
480 CCAAGTCTATCAATGACAACA-3' and 5'-CGCACTACATTAGTTCCAAT-3', respectively.
481 The sequence for generating the mock control shRNA was TTCTCCGAACGTGTCACGT.
482 These shRNAs were ligated into an AAV8 vector expressing H1 promoter and EGFP.

483

484 **Apoptotic percentage measurement**

485 After treatments, Hoechst 33342 (5 μ g/mL, Sigma-Aldrich; #B2261) and propidium iodide
486 (5 μ g/mL, Sigma-Aldrich; #P4170) were added simultaneously to each well to stain live
487 MSCs. Cell population was separated into 3 groups: healthy cells only showed a low level
488 of blue fluorescence; apoptotic cells showed a higher level of blue fluorescence, and dead
489 cells showed low-blue and high-red fluorescence. Stained cells were observed and
490 quantified by two independent experimenters without knowing the group assignment.
491 Results were expressed as a percentage of apoptosis (PA): PA = apoptotic cell number/
492 total cell number \times 100%.¹

493

494 **Measurement of oxidative stress in MSCs**

495 CellROX[®] oxidative stress reagent (Invitrogen, Carlsbad, CA; #C10444) is a novel
496 fluorogenic probe for measuring oxidative stress in living cells. After treatments, CellROX[®]
497 reagent was added at a final concentration of 5 μ M to MSCs, followed by incubation for 30
498 min at 37°C for fluorescence (green color) measurement by using an inverted fluorescent
499 microscope IX71 (Olympus microscope, Tokyo, Japan). Positive signals were quantified by
500 ImageJ software (Version 1.52r; NIH, Bethesda, MD).

501

502 **Detection of mitochondrial superoxide by flow cytometry**

503 After treatments, MSCs mitochondrial superoxide was measured with 5 μ M MitoSOX
504 (Invitrogen; #M36008) for 15 min at 37 °C. Cells were then washed with PBS, treated with
505 trypsin and resuspended in PBS containing 1% (v/v) heat-inactivated FBS. Data were
506 acquired with a FACS Calibur machine (BD Biosciences, San Jose, CA) and were
507 analyzed with the CellQuest analytical software.

508

509 **Serum and liver tissue processing and analysis**

510 After animal sacrifice, mouse serum was collected by centrifugation from whole blood
511 samples at 1,000x g for 10 min at 4°C and stored at -80°C. Serum ALT and AST levels
512 were measured by using ALT (SGPT; #A524-150) and AST (SGOT; #A559-150) reagent
513 sets (Teco diagnostics, Anaheim, CA) according to the manufacturer's instructions. Liver
514 tissue samples were fixed in 10% phosphate-buffered formalin, processed for histology
515 and embedded in paraffin blocks. Hepatic histology and fibrosis were visualized by
516 staining with hematoxylin/eosin (H&E) or Sirius Red using a LEICA Qwin Image Analyzer
517 (Leica Microsystems Ltd., Milton Keynes, UK).

518

519 **Western blotting, ELISA, and RT-PCR assays on key hepatic genes**

520 Protein extraction/quantification from MSCs or murine liver tissues, as well as Western

521 blotting assays were conducted as previously described³. Parallel blotting of GAPDH was
522 used as an internal loading control. TNF- α protein level was measured by using an ELISA
523 kit from PeproTech (#900-K25) according to the manufacturer's instructions. Activity
524 changes of Nrf2 (#TFEH-NRF2) were evaluated by using ELISA kits from RayBiotech
525 (Norcross, GA). Human NQO-1 (#ab28947) and HO-1 (#ab133064) protein level changes
526 were quantified by using ELISA kits from Abcam.

527

528 **Assay on transplantation safety**

529 To verify the long-term transplantation safety of MSCs in healthy and ACLF mice, we
530 performed a 24-week tumorigenicity study as previously described elsewhere⁵. Healthy
531 7-week-old C57BL/6J male mice (with or without ACLF induction) received 1×10^7 MRC-5
532 (negative control; ATCC, Manassas, VA; #CCL-171), 1×10^7 ES-D3 (positive control;
533 #CRL-11632), or 1×10^7 hADMSCs (MSCs group, with or without pIRES-Nrf2-DKK1
534 plasmid pre-transfection) (12 mice per group for healthy, 18 mice for ACLF group). After
535 24 weeks or when mice exhibited severe symptoms of dyspnea and minimal activity, mice
536 were sacrificed to assess the extent of tumor formation.

537

538 **Statistical Analysis**

539 Data from each group are presented as means \pm SD. Unless otherwise stated, statistical
540 comparisons between groups were done by using Kruskal-Wallis test, followed by Dunn's
541 post hoc test to determine differences in all groups. A value of $p < 0.05$ or less was
542 considered statistically significant (GraphPad Prism 5.0; San Diego, CA).

543

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549

550 **Author Contributions**

551 Hua Wang and Jia Xiao conceptualized the entire study and wrote the first draft of the
552 manuscript. Feng Chen and Zhaodi Che performed, and analyzed most experiments and
553 co-wrote the paper. Pingping Luo, Lu Xiao, Yali Song, Zhiyong Dong, Mianhuan Li, Min
554 Yang, Dongqing Wu and Yi Lv performed *in vitro* and *in vivo* experiments and analyzed
555 the results. Yingxia Liu, Cunchuan Wang, George L. Tipoe and Fei Wang analyzed the
556 results and revised the paper.

557

558 **Declaration of Interests**

559 The authors declare no competing interests.

560

561 **Additional Files**

562 **Supplementary files**

563 Supplementary file 1: Tumor incidence rate after transplantation of hADMSCs in healthy
564 and ACLF mice.

565 Supplementary file 2: Sequences of Plasmid Constructs

566 Transparent reporting form

567 **Data availability**

568 All data supporting the findings of this study are available within the article and its
569 supplementary files. Source data files have been provided for Figures 1 to 6.

570

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708

709 **Figure legends**

710 **Figure 1. Co-stimulation of MAPK/Nrf2 and Nanog/DKK1 signaling boosts**

711 **anti-stress capacity of human MSCs.** (A) Left: Changes of Nrf2 activity in MSCs with or

712 without TNF- α /H₂O₂ co-treatment. Right: Representative immunoblot results for DKK1

713 and secreted DKK1 (sDKK1) in MSCs with a similar test design as in panel A (*n* = 6). (B)

714 Left: Bioinformatic analysis identified a putative ARE (antioxidant response element)

715 located in the DKK1 promoter between -96 to -88 bp of the transcription start site (TSS).

716 Green boxes indicate DKK1 exons. The consensus sequence for the extended ARE is

717 shown, with the commonly identified core ARE indicated by the underlined sequence.

718 Abbreviations used follow standard IUPAC nomenclature (M = A or C; R = A or G; Y = C or

719 T; n = any nucleotide). Right: Relative luciferase activity of DKK1-ARE-WT and

720 DKK1-ARE-Mut (mutant) reporter vectors in the HEK-293T cells transfected with

721 pcDNA3.1-Nrf2 plasmid (*n* = 3). (C) Changes of DKK1 luciferase activity when Nrf2 was

722 knocked-down by siRNA with indicated concentrations (*n* = 6). (D) Experimental design

723 illustration showing that Nrf2 and DKK1 was overexpressed (OE) or knocked-down (KD)

724 by transfection with gene open reading frame-bearing plasmid or shRNA, respectively, at

725 the time of 48-h before the treatment of TNF- α /H₂O₂ in MSCs. (E) Changes in cell viability

726 and apoptotic ratios of MSCs following challenge with TNF- α /H₂O₂ in the presence or

727 absence of Nrf2/DKK1 manipulations (*n* = 6). (F) Representative immunoblot results for

728 PCNA and Bcl-2 from MSCs following the aforementioned treatments. (G) Changes in

729 cellular caspase-3/8 activity of MSCs with the aforementioned treatments (*n* = 6). (H) Left:

730 Fluorescence micrographs for the detection of cellular ROS by CellROX Green and

731 corresponding quantified fluorescence intensities in MSCs treated with TNF- α /H₂O₂ with
732 the aforementioned treatments ($n = 6$). Scale bar = 50 μ M. (i) Changes in mitochondrial
733 superoxide levels in MSCs measured by MitoSOX in flow cytometry, following the
734 aforementioned treatments. hADMSCs, human adipose-derived mesenchymal stromal
735 cells. Values are expressed as mean \pm SD. *, **, *** indicate $p < 0.05, 0.01, 0.001$ against
736 an untreated MSC group (or between indicated groups), respectively; #, ##, ### indicate $p <$
737 0.05, 0.01, 0.001 against an TNF- α /H₂O₂ group, respectively.

738

739 **Figure 2. Enhanced MSCs resilience through co-stimulation of MAPK/Nrf2 and**
740 **DKK1 signaling is attained by reducing ROS generation.** (A) Cell viability and
741 apoptotic ratios changes of MSCs treated with TNF- α /H₂O₂ in the presence or absence of
742 MitoQ or NAC ($n = 6$). (B) Representative immunoblotting results for PCNA and Bcl-2 in
743 MSCs (left) and changes in cellular caspase-3/8 activity of MSCs with a similar test design
744 as in panel A ($n = 6$). (C) (Left) Fluorescence micrographs for the detection of cellular ROS
745 by CellROX Green and corresponding quantified fluorescence intensities in MSCs
746 following antioxidant intervention ($n = 6$) (Scale bar = 50 μ M). (Right) Changes in
747 mitochondrial superoxide levels in MSCs following antioxidant intervention, as measured
748 by MitoSOX in flow cytometry ($n = 6$). (D) Assays on transcriptional activities of Nrf2 (left),
749 and representative immunoblot results (right) for phosphorylated p38 MAPK (p-p38), total
750 p38 MAPK (t-p38), p-ERK, t-ERK, and DKK1 in MSCs following antioxidant intervention (n
751 = 6). (E) (Left) Changes in transcriptional activities of Nrf2 in MSCs following TNF- α /H₂O₂
752 challenge in the presence or absence of MAPK/ERK inhibitors ($n = 6$). (Right-upper)

753 Representative immunoblot results for DKK1 and secreted DKK1 (sDKK1) in MSCs
754 following the aforementioned treatments. (Right-lower) Representative immunoblot results
755 for p-p38, t-p38, p-ERK, t-ERK of MSCs following TNF- α /H₂O₂ challenge, with or without
756 Nrf2/DKK1 overexpression (OE). Data are expressed as mean \pm SD. **, *** indicate $p <$
757 0.01 and $p <$ 0.001 against an untreated MSC group, respectively; #, ##, ### indicate $p <$
758 0.05, 0.01, 0.001 against a TNF- α /H₂O₂ group, respectively. SB, SB203580, the inhibitor
759 of p38 MAPK; U0, U0126, the inhibitor of ERK.

760

761 **Figure 3. Preconditioning by co-overexpression of Nrf2 and DKK1 enhances MSC**
762 **resistance to exogenous stress.** (A) Plasmid map for the constructed
763 pIRES2-Nrf2-DKK1. (B) Representative immunoblot results for time-lapse study (day 0 -
764 day 5) on Nrf2, DKK1 and secreted DKK1 (sDKK1) expression in MSCs following
765 transfection of the pIRES2-Nrf2-DKK1 plasmid. (C) Changes in cell viability (Left) and
766 apoptotic ratios (Right) of MSCs following TNF- α /H₂O₂ challenge with or without
767 transfection of the pIRES2-Nrf2-DKK1 plasmid ($n = 6$). (D) (Left) Representative
768 immunoblotting results for PCNA and Bcl-2 in MSCs and (Right) changes in cellular
769 caspase-3/8 activity of MSCs following TNF- α /H₂O₂ challenge with or without transfection
770 of the pIRES2-Nrf2-DKK1 plasmid ($n = 6$). (E) (Left) Detection of cellular ROS in MSCs by
771 CellROX Green and corresponding quantified fluorescence intensities in MSCs following
772 TNF- α /H₂O₂ challenge with or without transfection of the pIRES2-Nrf2-DKK1 plasmid ($n =$
773 6) (Scale bar = 50 μ M). (Right) Changes in mitochondrial superoxide levels in MSCs,
774 following TNF- α /H₂O₂ challenge with or without transfection of the pIRES2-Nrf2-DKK1

775 plasmid ($n = 6$). Data are expressed as mean \pm SD. *** indicates $p < 0.001$ against an
776 untreated MSC group; ### indicates $p < 0.001$ against an TNF- α /H₂O₂ group.

777

778 **Figure 4. Preconditioning with a pIRES2-Nrf2-DKK1 plasmid improves**
779 **transplantation efficacy of MSCs in a murine model of acute-on-chronic liver failure**

780 **(ACLF).** (A) Schematic timeline of the ACLF mice model establishment with carbon

781 tetrachloride (CCl₄) and *Klebsiella pneumoniae* (K.P.) injection with or without MSC of

782 Nrf2/DKK1 co-stimulation. (B) Survival counts of ACLF mice with or without injection of

783 human MSCs (naive or pre-transfected with pIRES2-Nrf2-DKK1) for 9 days ($n = 10$ per

784 group). (C) Changes in serum ALT levels in mice as depicted in panel A (72-h post *K.P.*

785 administration; $n = 8$). (D) Changes in liver total bilirubin (TBIL), circulating neutrophils,

786 blood urea nitrogen (BUN), and renal microvascular flow in mice as depicted in panel A

787 (72-h post *K.P.* administration; $n = 6$). (E) Representative images of H&E and Sirius Red

788 staining of the mice liver, and corresponding quantification of liver necrosis areas and liver

789 fibrosis areas, and changes in liver MDA contents in mice as depicted in panel A (72-h

790 post *K.P.* administration; $n = 6$). (F) Representative immunohistochemical results for

791 human nuclear antigen (hNA) and Ki67 staining and representative immunoblot results for

792 human DKK1/ α AT in the liver from mice as depicted in panel A (72-h post *K.P.*

793 administration; $n = 6$). Scale bar = 50 μ M. Arrows indicate typical IHC signals. Data are

794 expressed as mean \pm SD. **, *** indicate $p < 0.01$, 0.001 against a healthy group,

795 respectively; #, ##, ### indicate $p < 0.05$, 0.01, 0.001 against an ACLF group, respectively;

796 @, @@, @@@ indicate $p < 0.05$, 0.01, 0.001 against an ACLF-challenged plasmid-naïve

797 MSC group, respectively.

798

799 **Figure 5. MSCs preconditioned with pIRES2-Nrf2-DKK1 resolves ACLF injury by**
800 **enhancing the pro-regenerative IL-6/STAT3 pathway but attenuating the**
801 **anti-regenerative IFN- γ /STAT1 pathway.** (A,B) Serum IL-6 and IFN- γ , and relative
802 mRNA expressions of *Il-6* and *Irfng*, and their downstream target genes (*Bcl2* and *Stat1*,
803 respectively) in the ACLF or control mice after the transplantation with MSCs, with or
804 without pIRES2-Nrf2-DKK1 plasmid preconditioning ($n = 6$). (C) Liver extracts were
805 subjected to Western blot analysis of phosphorylated STAT1 (pSTAT1), STAT1, pSTAT3,
806 STAT3 and Cyclin D1 in mice as depicted in panel A. (D) Relative quantification of STAT1
807 and STAT3 (the phosphorylated level was divided by the total protein level) and Cyclin D1
808 in mice as depicted in panel A ($n = 3$). Data are expressed as mean \pm SD. *, **, *** indicate
809 $p < 0.05, 0.01, 0.001$ against a healthy group, respectively; #, ##, ### indicate $p < 0.05, 0.01,$
810 0.001 against an ACLF group, respectively; @@@ indicates $p < 0.001$ against an
811 ACLF-challenged plasmid-naïve MSC group.

812

813 **Figure 6. The DKK1 receptor CKAP4, but not LRP6, in host hepatocytes is a**
814 **paracrine target of MSC-based therapy.** (A) Representative genotyping results for
815 hepatic-specific knockdown of CKAP4 and LRP6 by AAV8-mediated shRNA in mice (in
816 the tissue extracts of bone marrow, lung, heart, kidneys, brain, and liver). (B) Survival
817 counts of mice with ACLF challenge with or without injection of human MSCs (naïve or

818 pre-transfected with pIRES2-Nrf2-DKK1) for 9 days, following hepatic knockdown (CKD)
819 of CKAP4 or LRP6 ($n = 10$ per group). (C) Changes in serum ALT levels in mice as
820 depicted in panel B (72-h post *K.P.* administration; $n = 6$). (D) Representative images for
821 liver H&E staining and corresponding quantification of liver necrosis areas and liver
822 fibrosis areas, and changes in liver MDA contents in mice as depicted in panel B (Dashed
823 lines indicate typical necrotic areas in the liver. 72-h post *K.P.* administration; $n = 6$). (E)
824 Representative images for liver human nuclear antigen (hNA) immunohistochemical
825 staining and corresponding quantification of hNA density in mice as depicted in panel B
826 (72-h post *K.P.* administration; $n = 6$). Arrows indicate typical IHC signals. (F)
827 Representative images for liver Ki67 immunohistochemical staining and corresponding
828 quantification of Ki67 density, and enzyme-immuno assay measurements of hepatic Akt
829 activity in mice as depicted in panel B ($n = 6$). (G) Representative immunoblot results for
830 liver phosphorylated Akt (p-Akt), Akt, cyclin D1, and PCNA in mice with or without
831 knockdown of hepatic CKAP4 or LRP6. Scale bar = 50 μ M. Data are expressed as mean
832 \pm SD. *** indicates $p < 0.001$ against a healthy group; ##, ### indicate $p < =0.01, 0.001$
833 against an ACLF group, respectively; @, @@, @@@ indicate $p < 0.05, 0.01, 0.001$ against an
834 ACLF-challenged plasmid-naïve MSC group, respectively. For panels E and F, *, **, ***
835 represent $p < 0.05, 0.01, 0.001$ between indicated groups.
836

837 **Figure supplement 1. Validation of cell surface markers.** Flow cytometry analysis on
838 human adipose-derived mesenchymal stromal cells (hADMSCs) showed their high
839 expression in CD44 and CD105 and low expression in CD34 and CD45.

840

841 **Figure 1- figure supplement 2. ELISA results of released IL-1 β , IL-6, MCP-1, RANTES,**
842 **and IL-10 protein from MSCs treated with TNF- α /H₂O₂ (n = 6).** Data are expressed as
843 mean \pm SD. *** indicates $p < 0.001$ against the control group.

844

845 **Figure 2- figure supplement 3. Regulatory loop between Nrf2/DKK1 and**
846 **NQO-1/HO-1 in MSCs.** (A) Changes in NQO-1/HO-1 protein expression of MSCs
847 following TNF- α /H₂O₂ challenge in the absence or presence of Nrf2/Dkk1 manipulations
848 ($n = 6$). (B) Representative immunoblot results for MSCs when endogenous NQO-1/HO-1
849 was inhibited specifically by shRNAs. (C) Changes in cell viability or apoptotic ratios of
850 MSCs following TNF- α /H₂O₂ challenge in the presence or absence of NQO-1/HO-1
851 inhibition or Nrf2/DKK1 overexpression (OE) ($n = 6$). Data are expressed as mean \pm SD.
852 For panels A and B, *** indicates $p < 0.001$ against an untreated MSC group; #, ##, ###
853 indicate $p < 0.05, 0.01, 0.001$ against a corresponding TNF- α /H₂O₂ group, respectively.
854 For panel C, *, **, *** represent $p < 0.05, 0.01, 0.001$ between indicated groups,
855 respectively.

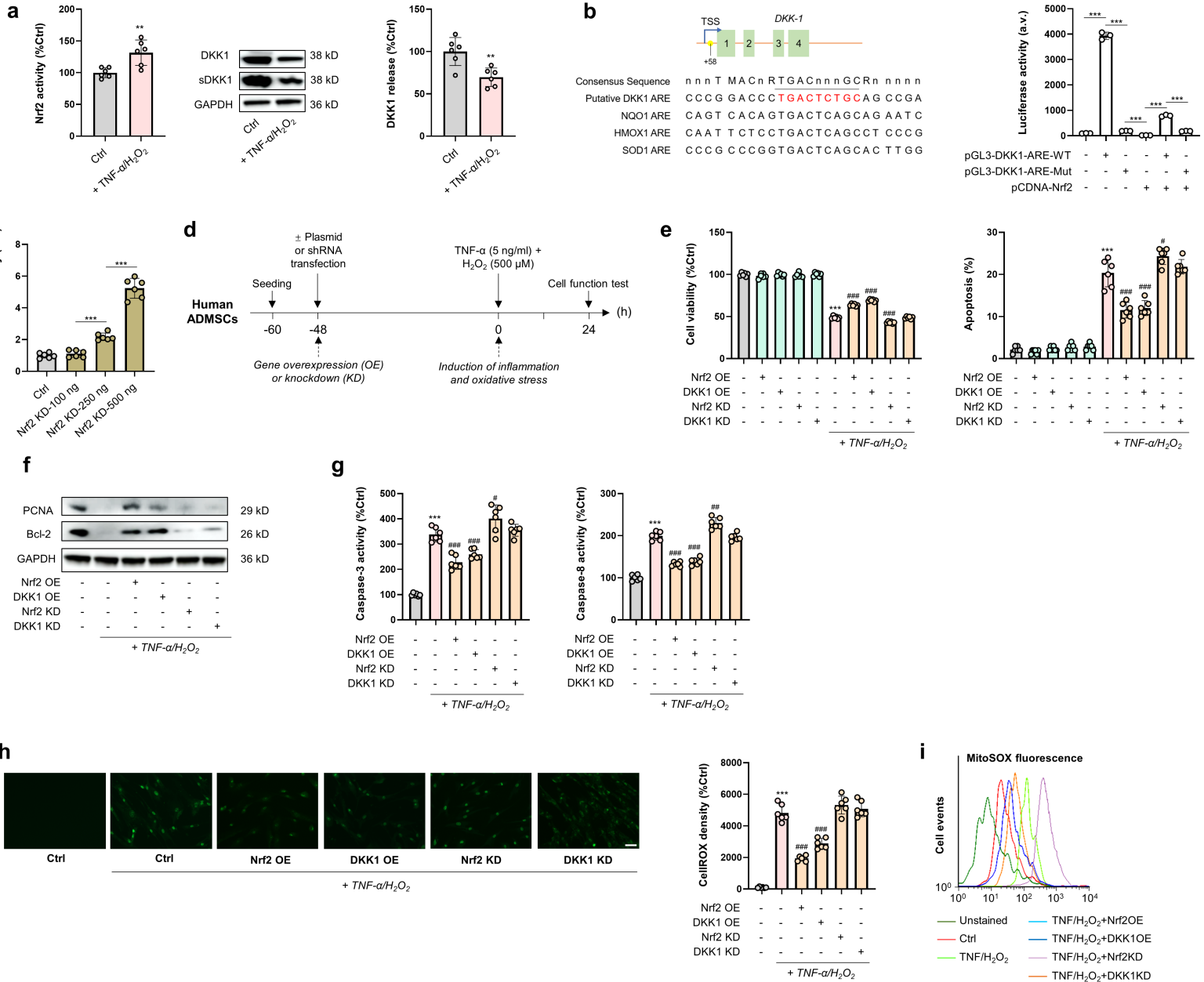
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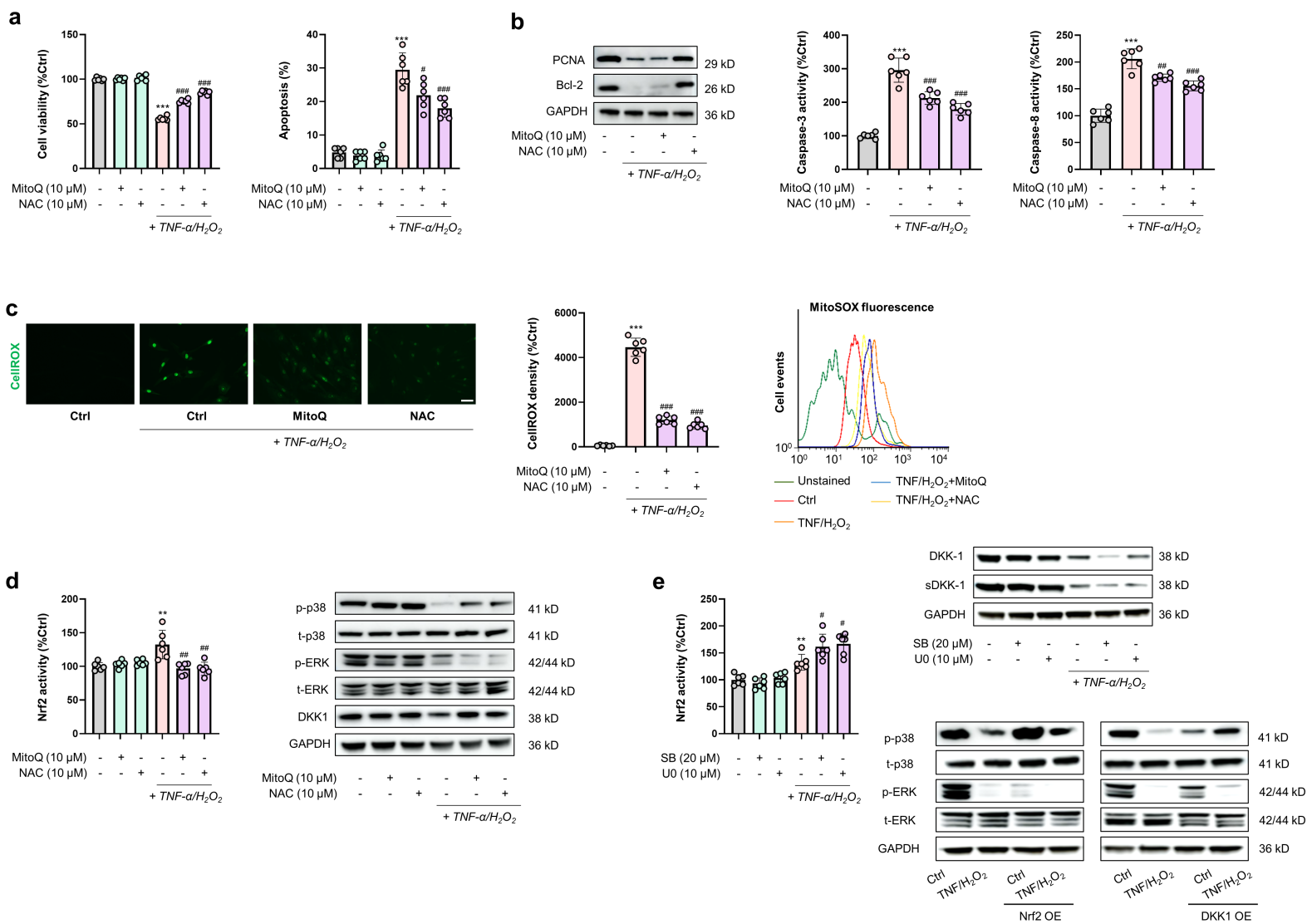
857 **Figure 3- figure supplement 4. Transfection with a pIRES2-Nrf2-DKK-1 plasmid does**
858 **not interfere with MSCs transdifferentiating potential or cell status.** (A)
859 Representative images of MSCs adipogenic, osteogenic, and chondrogenic differentiation
860 by using commercial standardized protocols. (B) Quantitative RT-PCR measurements of
861 key genes for MSCs adipogenic, osteogenic, and chondrogenic differentiation. (C)
862 Changes in MSCs viability, apoptosis ratios, and caspase-3/7 activities on day 0-5

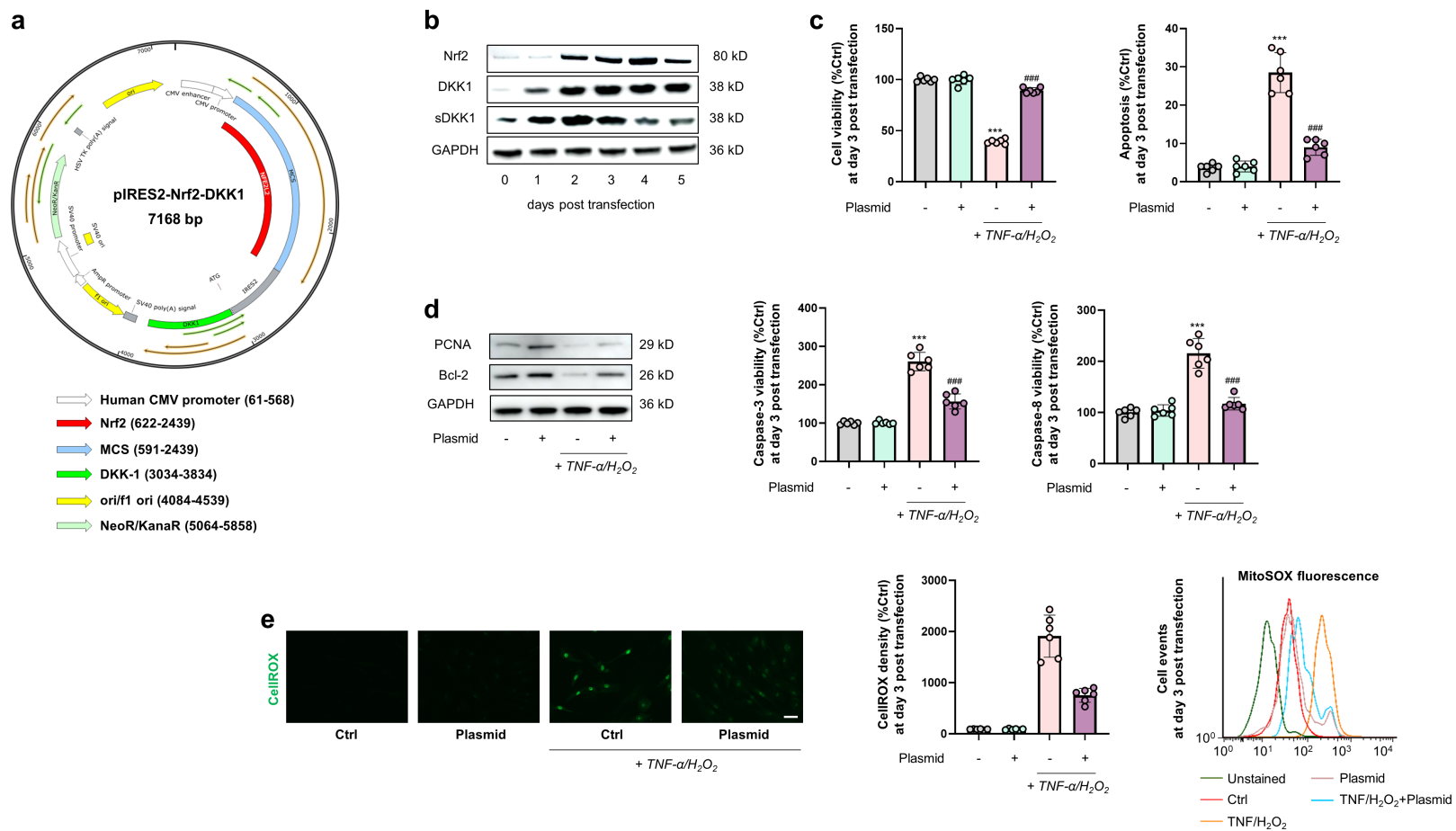
863 following transfection with the pIRES2-Nrf2-DKK1 plasmid. All data shown herein are that
864 of MSCs. Data are expressed as mean \pm SD. *, ** indicate $p < 0.05$, 0.01 against a
865 corresponding untreated MSCs group, respectively.

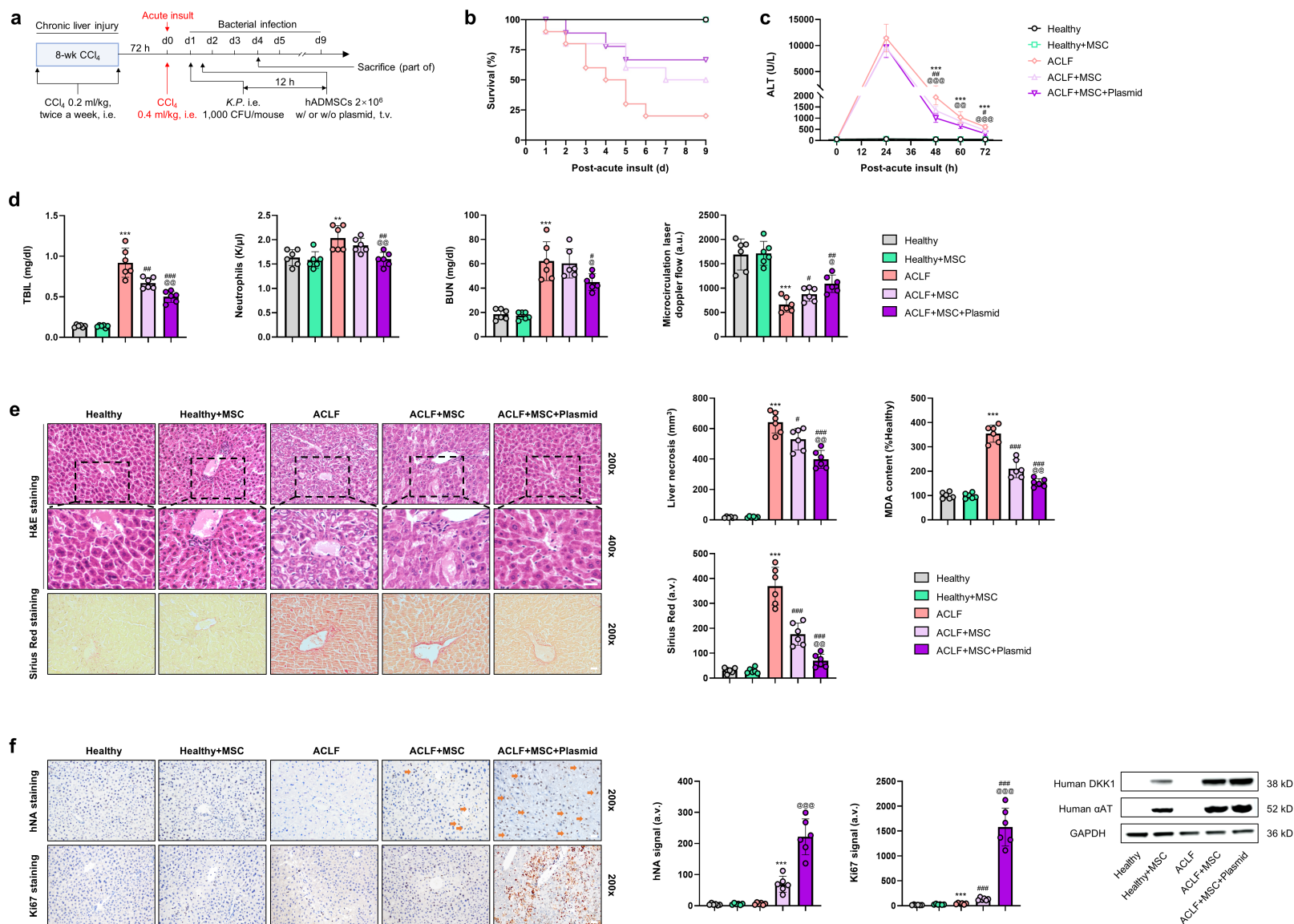
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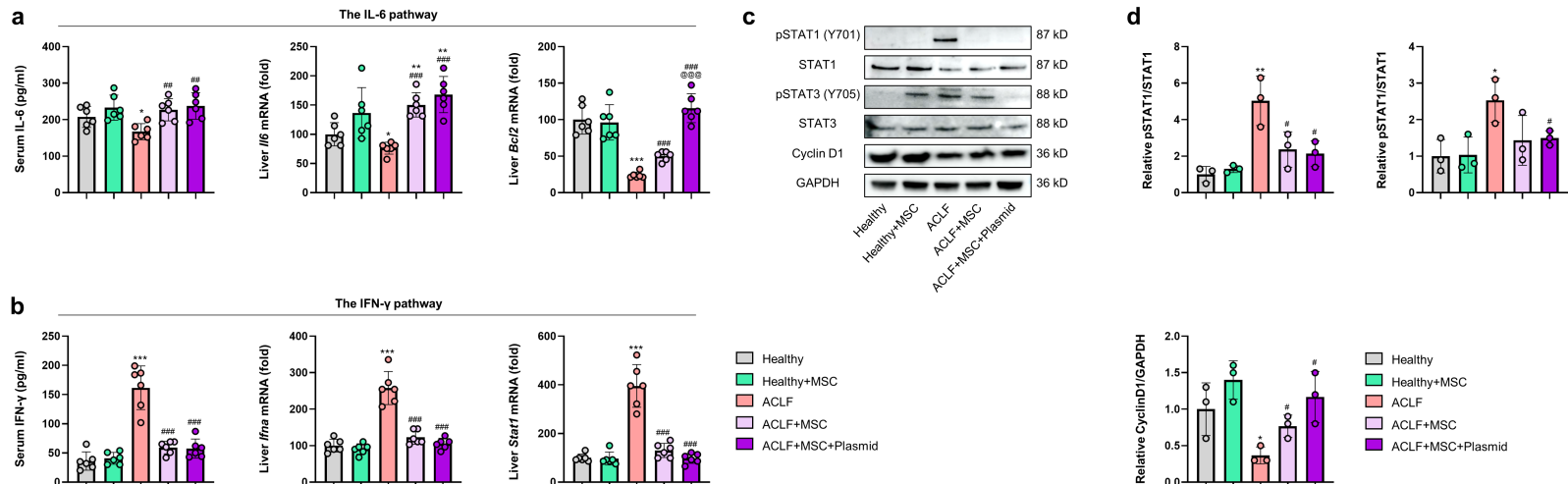
867 **Figure supplement 5. Long-term (12- and 24-week) donor cell function in healthy**
868 **and ACLF mice transplanted with human MSCs with or without plasmid**
869 **pre-transfection.** Human albumin was determined in the serum by an ELISA assay at
870 weeks 12 and 24 for healthy or ACLF mice ($n = 6$) transplanted with preconditioned MSCs.
871 Mice without MSC transplantation served as negative controls. Data are expressed as
872 mean \pm SD. Results are representative of at least 3 independent experiments. *, **, ***
873 represent $p < 0.05$, 0.01 , 0.001 between indicated groups.

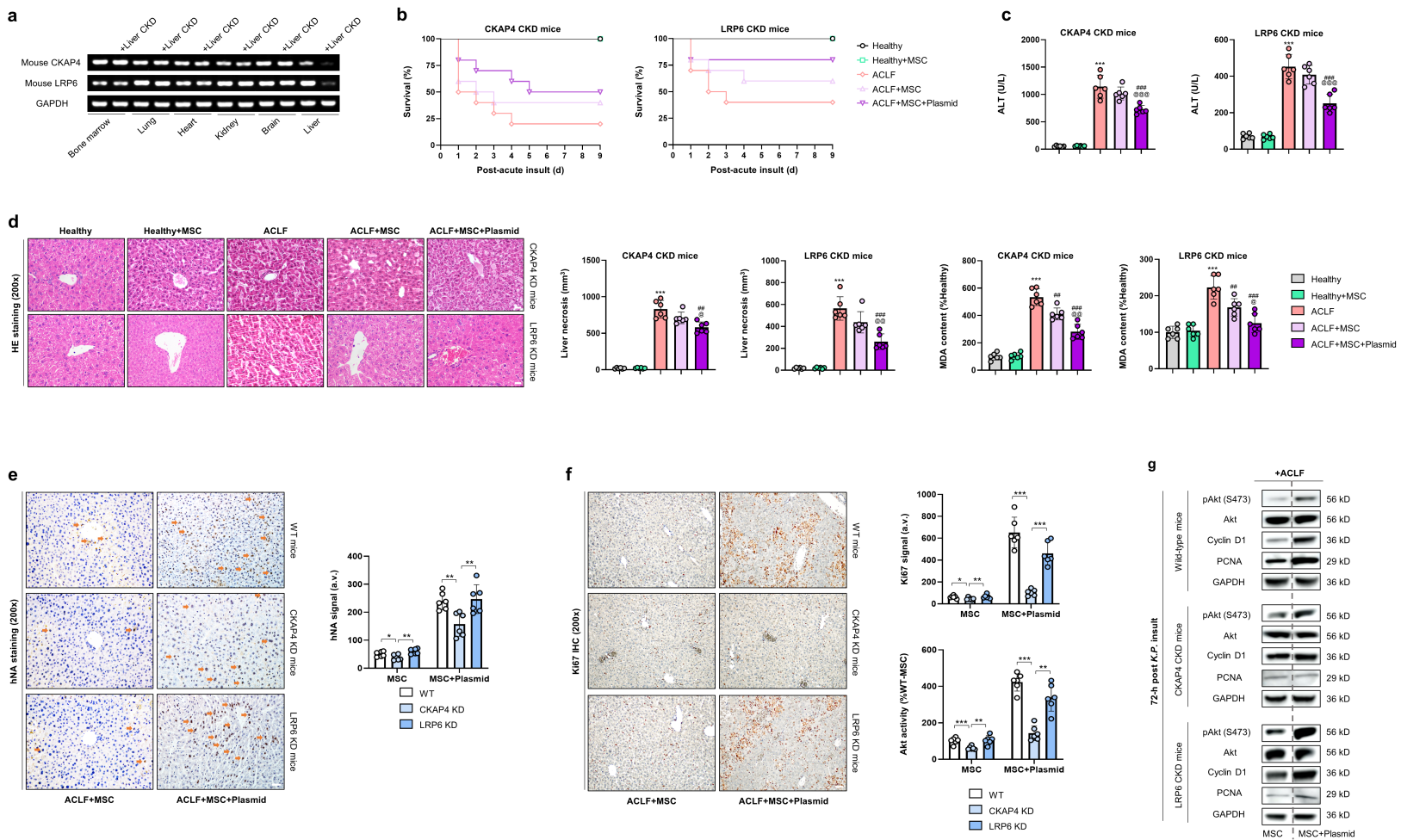


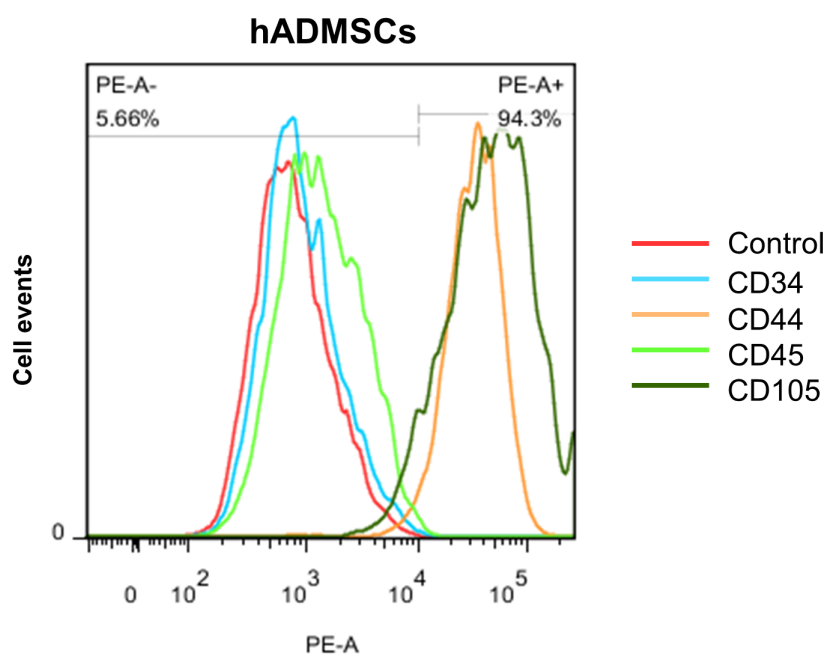












Marker	hADMSCs
Control	0.1%
CD34	0.3%
CD44	0.5%
CD45	97.6%
CD105	94.3%

