LDHA-mediated metabolic reprogramming promoted cardiomyocyte proliferation by alleviating ROS and inducing M2 macrophage polarization

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\textbf{A R T I C L E  I N F O}

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\textbf{A B S T R A C T}

Aims: Metabolic switching during heart development contributes to postnatal cardiomyocyte (CM) cell cycle exit and loss of regenerative capacity in the mammalian heart. Metabolic control has potential for developing effective CM proliferation strategies. We sought to determine whether lactate dehydrogenase A (LDHA) regulated CM proliferation by inducing metabolic reprogramming.

Methods and results: LDHA expression was high in P1 hearts and significantly decreased during postnatal heart development. CM-specific LDHA knockout mice were generated using CRISPR/Cas9 technology. CM-specific LDHA knockout inhibited CM proliferation, leading to worse cardiac function and a lower survival rate in the neonatal apical resection model. In contrast, CM-specific overexpression of LDHA promoted CM proliferation and cardiac repair post-MI. The α-MHC-H2B-mCh/CAG-eGFP-anillin system was used to confirm the proliferative effect triggered by LDHA on P7 CMs and adult hearts. Metabolomics, proteomics and Co-IP experiments indicated that LDHA-mediated succinyl coenzyme A reduction inhibited succinylation-dependent ubiquitination of thioredoxin reductase 1 (Txnrd1), which alleviated ROS and thereby promoted CM proliferation. In addition, flow cytometry and western blotting showed that LDHA-driven lactate production created a beneficial cardiac regenerative microenvironment by inducing M2 macrophage polarization.

Conclusions: LDHA-mediated metabolic reprogramming promoted CM proliferation by alleviating ROS and inducing M2 macrophage polarization, indicating that LDHA might be an effective target for promoting cardiac repair post-MI.

1. Introduction

Mammalian cardiomyocytes (CMs) have a remarkable regenerative capacity in the fetal stage and quickly lose this potential in the first week after birth [1]. The loss of the CM proliferative capability was associated with the metabolic switch from anaerobic glycolysis to oxidative phosphorylation [2,3]. In the fetal heart, glycolysis is a major source of energy for proliferating CMs [3]. However, by 7 days after birth, glycolysis decreases and oxygen-dependent mitochondrial oxidative phosphorylation becomes a major source of energy in the adult heart [3]. This metabolic switching results in an increase in intracellular reactive oxygen species (ROS) levels and subsequent DNA damage in CM, which contributes to postnatal CM cell cycle arrest [4]. Since there

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is a strong association between the metabolic switch and CM proliferative capacity, metabolic control in the postnatal heart has tremendous potential for developing new CM proliferation strategies.

Many approaches have been demonstrated to achieve metabolic control in the postnatal heart, including the regulation of metabolic enzymes or metabolites. Glycolytic enzymes Glut1 and Pkm2 over-expression promoted CM proliferation by increasing nucleotide biosynthesis and activating β-catenin pathways, respectively [5,6]. Targeting the metabolites involved in oxidative phosphorylation, such as succinate and fatty acids, could extend the time window of postnatal CM proliferation by reducing ROS generation [3,7]. These studies indicated that metabolic enzymes and metabolites play important roles in CM proliferation. Lactate dehydrogenase A (LDHA), a metabolic enzyme that catalyzes the conversion of pyruvate to lactate in the glycolytic pathway [8], has been reported to regulate cell proliferation. LDHA inhibition slowed liver and breast cancer progression [9,10] while glycolytic pathway [8], has been reported to regulate cell proliferation.

Posttranslational modifications (PTMs) are important regulators of protein function, and provide a mechanism to integrate metabolism with physiological and pathological processes [13]. Succinylation is a recently discovered and meagerly studied PTM in which metabolically derived succinyl coenzyme A (succ-CoA) to modify protein lysine groups [13]. Succinylation coordinates metabolism and signaling pathways by utilizing the metabolic intermediate succ-CoA as a sensor [13]. Suc-CoA is a product mainly converted from acetyl-CoA in the heart [15], and succinylation was identified as an important regulator of cardiac metabolism and function [15]. Accumulating evidence demonstrated that LDHA was involved in metabolite-dependent protein PTMs such as acetylation [16] and phosphorylation [17]. Therefore, we postulated that succinylation might establish a link between LDHA and CM proliferation.

Consistent with this hypothesis, we found that LDHA reduced the intracellular succ-CoA concentration to inhibit succinylation-dependent ubiquitination of Txnrd1, thereby increasing Txnrd1 protein levels and subsequently promoting ROS reduction and CM proliferation. LDHA has been reported to increase lactate production [8] and lactate could promote muscle regeneration after injury by inducing M2 macrophage polarization [18], whether cardiomyocyte LDHA-driven lactate production is beneficial for cardiac regeneration post-MI remains unknown. Our study also revealed that LDHA-driven lactate created a beneficial cardiac regenerative microenvironment by inducing M2 macrophage polarization. These findings indicated that targeting metabolic reprogramming through LDHA to alleviate ROS and induce M2 macrophage polarization might be an effective CM proliferation strategy.

2. Materials and methods

Healthy C57BL/6 N mice were provided by Southern Medical University. A Cre-dependent Cas9 knock-in mouse model (R26-CAG-Cas9/+ ) was purchased from GemPharmatech, Jiangsu, China. α-MHC-Cre mice were provided by Dr. Kunfu Ouyang from Peking University Shenzhen Graduate School, China. αHMC-H2B-mCh and CAG-eGFP-anillin mice were provided by Dr. Michael Hesse from University of Bonn, Germany. All mice were provided ad libitum access to water and food in a specific-pathogen-free facility under standard conditions and monitored daily for health and activities. Mice from different experimental groups were housed together in cages on 12-h light/dark cycles. Adult male mice (6-8 weeks) were chosen for adult experiments. Adult male mice have a higher susceptibility to cardiac dysfunction in response to left anterior descending artery ligation, which allows the detection of changes associated with CM proliferation without confounding factors when compared with adult female mice [19]. All groups were blinded to genotype until data analysis was completed, and all experimental procedures were conformed to the guidelines of Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Southern Medical University Institutional Animal Care and Use Committee.

2.1. Establishment of the α-MHC-H2B-mCh/CAG-eGFP-anillin system

The α-MHC-H2B-mCh/CAG-eGFP-anillin double transgenic mouse line was produced by crossing the α-MHC-H2BmCh-mice with the CAG-eGFP-anillin-mice [20-22]. The double transgenic mice (α-MHC-H2BmCh-mice/CAG-eGFP-anillin) were maintained on a mixed genetic background (CD-1 × C57BL/6Ncr × 129S6/SvEvTac). This double transgenic mouse line enables the unequivocal identification of CMs based on mCh fluorescence and the cell cycle status, as indicated by eGFP-anillin expression and its subcellular localization. Since the anillin protein is localized in the nucleus during G1/S/G2 phases of the cell cycle and translocates to the cytoplasm in M-phase and resides in the contractile ring and midbody during cytokinesis, this eGFP-anillin system enables the visualization of cell cytokinesis with high spatiotemporal resolution.

2.2. Statistical analysis

All data were presented as the means±SDs, and the results were analyzed with SPSS 18.0 software. All continuous variables were subjected to a normality test. An unpaired Student’s t-test was used to

| Abbreviations | Description |
|---------------|-------------|
| AAV9          | (adeno-associated virus 9) |
| Ac-CoA        | (acetyl coenzyme A) |
| AMI           | (acute myocardial infarction) |
| AR            | (apical resection) |
| CMs           | (cardiomyocytes) |
| D-FBP         | (d-fructose 1,6-bisphosphate) |
| D-G6P         | (d-glucose 6-phosphate) |
| DHAP          | (dihydroxyacetone phosphate) |
| DHE           | (dihydroethidium) |
| dpar          | (days post-apical resection) |
| LDHA          | (lactate dehydrogenase A) |
| NHE           | (Na+/H+ exchanger) |
| NCX           | (Na+/Ca2+ exchanger) |
| OAA           | (oxaloacetate) |
| pH3           | (phosphorylated histone H3) |
| PTMs          | (posttranslational modifications) |
| ROS           | (reactive oxygen species) |
| siRNAs        | (small interfering RNAs) |
| Succ-CoA      | (succinyl coenzyme A) |
| TPP           | (thiamine pyrophosphate) |
| Txmr1d        | (thioredoxin reductase 1) |
| 8-OHG         | (8-hydroxyguanosine) |
| β-F6P         | (beta-o-fructose-6-phosphate) |
| α-KGDC        | (α-ketoglutarate dehydrogenase complex) |
analyze the two groups. One-way ANOVA followed by Bonferroni’s multiple comparisons test or two-way ANOVA followed by Sidak’s test were used to analyze the multiple groups. For variables with an abnormal distribution, the nonparametric Mann–Whitney U test was used to compare the two independent groups. The survival rate was determined by using the Kaplan–Meier method, and differences between survival curves were determined using the log-rank (Mantel–Cox) test. $P < 0.05$ was considered statistically significant. More detailed

Fig. 1. LDHA was involved in neonatal cardiac regeneration after injury. (A–D) LDHA protein levels in mouse hearts at different ages and neonatal hearts harvested at 2 and 7 dpar. $n = 6$ mice in A and C; $n = 5$ mice in B and D. (E) Scheme depicting the cross-breeding of the α-MHC-Cre mice with the Cas9-tdTomato mice to obtain mice myocardially expressing Cas9, followed by the delivery of Adv-expressing sgRNA. (F) Masson staining of neonatal hearts harvested at 14 dpar. $n = 13$ mice in Cre-empty and $n = 10$ mice in Cre-LDHA groups. (G–H) Analysis of cardiac function and survival rate after LDHA deficiency. $n = 8$ mice in G; $n = 30$ mice in H. Bars $= 20 \mu m$ in B and D, and 500 $\mu m$ (upper) and 100 $\mu m$ (lower) in F. Statistical significance was calculated using an unpaired t-test in F, one-way ANOVA in A–D and the log-rank (Mantel–Cox) test in H; *$P < 0.05$. 
descriptions of the materials and methods are provided in the Supplementary Information.

3. Results

3.1. LDHA was involved in neonatal cardiac regeneration after injury

We first observed that LDHA levels decreased significantly during postnatal heart development (Fig. 1A–B) and increased partially at 2 and 7 days post-apical resection (dpar) in neonatal hearts (Fig. 1C–D). Next, LDHA was knocked down in isolated P1 CMs selected at approximately 87% purity (Fig. S1A). We found that LDHA expression was decreased (Figs. S1B–C) and LDHA deficiency inhibited cell cycle re-entry of P1 CM under physiological condition, as stained by the cell cycle markers Ki67, pH3 and aurora B (Figs. S1D–F).

We found that LDHA could be detected in different cells and was highly expressed in CMs after analyzing the single-cell RNA-seq in neonatal hearts (GSE153481) and qRT-PCR further confirmed the data (Figs. S2A–B). Hence, we constructed CM-specific LDHA knockout mice by injecting Adv-sgRNA (LDHA) into neonatal mouse hearts with myocardial Cas9-tdTTomato expression (Fig. 1E and S2C–D). Adv-sgRNA (LDHA) was successfully transduced and LDHA expression was significantly decreased (Figs. S2E–G). CM-specific LDHA deficiency hindered CM proliferation (Figs. S3A–C), leading to markedly increased fibrosis, worse cardiac function and a lower survival rate at 14 dpar (Fig. 1F–H). Overall, these data implied that cardiomyocyte LDHA was involved in neonatal cardiac regeneration.

3.2. LDHA overexpression promoted P7 CM proliferation in vitro

To determine whether LDHA could promote P7 CM proliferation, we delivered an adenoviral-mediated LDHA transfer vector in isolated P7 CMs selected at approximately 80% purity (Fig. S4A). Adv-LDHA was successfully transduced and LDHA expression was significantly increased (Figs. S4B–D). LDHA overexpression promoted cell cycle re-entry of P7 CMs under physiological condition (Fig. 2A–C), induced cell division (Fig. 2D and Video 1) and resulted in an increase in the percentage of mononucleated CMs (Fig. 2E). We then used the α-MHC-H2B-mCh/CAG-eGFP-anillin system to further confirm that LDHA overexpression promoted CM proliferation (Fig. 2E and S4F). LDHA overexpression in isolated P7 CMs from α-MHC-H2B-mCh/CAG-eGFP-anillin double transgenic mice led to an increase of regular midbodies, as stained by Anillin and aurora B (Fig. 2G–I). These data indicated that LDHA overexpression promoted CM cytokinesis in addition to cell cycle re-entry.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.redox.2022.102446

3.3. LDHA overexpression promoted adult CM proliferation in vivo

To investigate the effect of LDHA in adult hearts, AAV9 vector carrying the LDHA gene with CM-specific cTnT promoter (AAV9-cTnT-LDHA) were injected in normal adult hearts (Figs. S5A–B). LDHA levels were increased (Figs. S5C–D) and LDHA overexpression promoted CM cell cycle re-entry in adult hearts under physiological condition (Fig. 3A–C). Moreover, the proliferative effect triggered by LDHA was confirmed using CAG-eGFP-anillin transgenic mice, showing that approximately half of the adult cardiomyocytes underwent autocrine cell division (Fig. 3D). After isolating adult CMs from adult mouse hearts injected with AAV9-cTnT-LDHA or AAV9-cTnT-NC, we found that LDHA overexpression also induced an increase in the percentage of mononucleated CMs in vivo (Fig. 3E). Besides, LDHA overexpression slightly increased the heart/body weight ratio and adult CM size (Fig. 3F–G). Stereological analysis and hemocytometer cell counting method confirmed an increase of CM numbers after LDHA overexpression (Fig. 3H–J). Collectively, these results revealed that overexpression of LDHA promoted cardiomyocyte proliferation in normal adult hearts.

3.4. LDHA overexpression improved cardiac function after MI in adult mice

To evaluate whether LDHA could improve myocardial repair and preserve cardiac function after MI, AAV9-cTnT-LDHA was delivered into the peri-infarcted area in adult MI model (Figs. 5A and 4A). LDHA expression was increased after AAV9-cTnT-LDHA transduction (Figs. 5B–C). LDHA overexpression increased the proliferative rate of adult cardiomyocytes (Fig. 4B–D) and slightly increased the CM size in the peri-infarct zone (Fig. 4E). Moreover, LDHA overexpression significantly reduced the infarcted areas and improved cardiac function in adult mouse hearts post-MI (Fig. 4F–H).

3.5. LDHA overexpression induced metabolic reprogramming in CMs

We next explored whether LDHA could drive metabolic reprogramming to regulate CM proliferation. The targeted metabolomes, metabolic assaying and qRT-PCR results showed that LDHA overexpression increased the levels of several glycolytic related metabolites and genes, while decreased the levels of several TCA cycle metabolites and genes in P7 CMs (Fig. 5A–D and S7A–E), indicating that LDHA could drive metabolic reprogramming in CMs.

We further performed proteomics in P7 CMs after LDHA overexpression to detect the proteins that might be regulated by LDHA. 161 differentially expressed proteins were identified (Fig. 5E and Supplemental Table 4). GO enrichment analysis showed that differentially expressed proteins were mainly involved in the generation of precursor metabolites and energy, ROS and positive regulation of the mitotic cell cycle (Fig. 5F). KEGG enrichment analysis showed that they were significantly enriched in cell cycle, oxidative phosphorylation and ROS pathway (Fig. 5G). The Gene set enrichment analysis (GSEA) indicated that LDHA overexpression led to a dynamic bias towards positive regulation of CM proliferation and heatmap showed that LDHA overexpression upregulated several cell cycle genes including Cdk1, Cdk2, Cdc16 and Cdc23 (Fig. 5H–I). Moreover, LDHA upregulated the glucose metabolism-related proteins (Fig. 5J) and downregulated oxidative phosphorylation-related proteins (Fig. 5K). In conclusion, LDHA overexpression drove metabolic reprogramming, which has the potential to promote cardiac regeneration.

3.6. LDHA overexpression reduced ROS levels and oxidative stress in CMs

We sought to determine whether LDHA regulated CM proliferation by metabolically controlling ROS alleviation. LDHA downregulation increased the ROS and oxidative DNA damage levels in P1 CMs (Figs. S8A–B) while LDHA overexpression upregulated the levels of several antioxidant genes, reduced intracellular and mitochondrial ROS, inhibited oxidative DNA damage and inhibited DNA damage repose mediator p-ATM in P7 CMs (Fig. 6A–B and SBC-E). Furthermore, LDHA deficiency increased the ROS and oxidative DNA damage levels in neonatal AR hearts (Figs. S8F–G) while LDHA overexpression had opposite effect in normal adult hearts and the peri-infarct zone of adult MI hearts (Fig. 6C–F). We then performed rescue experiments in neonatal AR models using the antioxidant MitoQ to examine whether ROS are involved in LDHA deficiency-induced cell cycle arrest. MitoQ abrogated the increased levels of ROS and oxidative DNA damage, and partially abrogate the cell cycle arrest induced by LDHA deficiency in neonatal AR hearts (Fig. 6G–H and S8H). These results suggested that ROS played an important role in LDHA-mediated CM proliferation.

3.7. LDHA-mediated ROS alleviation promoted CM proliferation via Tnrd1

To explore how LDHA mediated ROS alleviation, we screened our
Fig. 2. LDHA overexpression promoted P7 CM proliferation in vitro.

(A) Ki67 staining in P7 CMs (559 CMs from 12 images of 6 mice in Adv-NC and 866 CMs from 12 images of 6 mice in Adv-LDHA groups). (B) pH3 staining in P7 CMs (1297 CMs from 24 images of 6 mice in Adv-NC and 1486 CMs from 24 images of 6 mice in Adv-LDHA groups). (C) Aurora B staining in P7 CMs (3342 CMs from 6 mice in Adv-NC and 3797 CMs from 6 mice in Adv-LDHA groups). (D) Time-lapse images of P7 CM cytokinesis (4479 CMs from 5 mice in Adv-NC and 4357 CMs from 5 mice in Adv-LDHA groups). (E) Analysis of CM cell nucleation in isolated P7 CMs (1963 CMs from 6 mice in Adv-NC and 2190 CMs from 6 mice in Adv-LDHA groups). (F) Scheme depicting the cross-breeding of α-MHC-H2B-mCh mice with the CAG-eGFP-anillin proliferation indicator mice. (G–I) Example of a P7 double transgenic CM expressing eGFP-anillin (green) and aurora B (white) after LDHA overexpression and the frequency of regular and irregular midbodies identified by anillin or aurora B staining (5103 CMs from 5 mice in Adv-NC and 2240 CMs from 5 mice in Adv-LDHA groups). Bars = 50 μm (left) and 20 μm (right) in A and B, 20 μm in C, E and G, and 50 μm in D. Statistical significance was calculated using an unpaired t-test in A–D and two-way ANOVA in E and H–I; *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
proteomics data and found that several downstream proteins were involved in the ROS metabolic process (Fig. 7A). Txnrd1, a key enzyme required for protection against oxidative stress, was the most significantly upregulated protein (Fig. 7B) and its expression gradually decreased during postnatal heart development (Fig. 7C). Moreover, Txnrd1 overexpression reduced ROS and promoted CM proliferation in both P7 CMs and adult hearts (Fig. 7D–E).

To investigate whether Txnrd1 is key for LDHA-mediated ROS alleviation and CM proliferation, we performed rescue experiments using the Txnrd1 inhibitor auranofin or si-Txnrd1. We tested multiple doses of auranofin or si-Txnrd1 abrogated the decreased ROS levels, decreased oxidative DNA damage and increased proliferative rate induced by LDHA (Figs. S10A–C, S11A–C and S12A–C). Taken together, these results indicated that LDHA-mediated ROS alleviation promoted CM proliferation via Txnrd1.

3.8. LDHA-mediated suc-CoA reduction inhibited succinylation-dependent ubiquitination of Txnrd1

We next investigated how LDHA overexpression increased the Txnrd1 protein level. We found that LDHA had no effect on Txnrd1 mRNA levels (Fig. 8A) but substantially prolonged the half-life of endogenous Txnrd1 in P7 CMs (Fig. 8B). Moreover, LDHA overexpression reduced Txnrd1 ubiquitination in P7 CMs (Fig. 8C).

Our targeted metabolomics data revealed that suc-CoA was the most significantly downregulated metabolite among the metabolites that could mediate PTMs (Fig. 8D–G). LDHA overexpression decreased the protein levels of the α-KGDC subunits OGDH, DLST and DLD (Figs. S13A–C). In addition, the targeted metabolomics and metabolic assays showed that LDHA overexpression reduced the intracellular levels of TPP, NAD+ and NADH (Figs. S13D–G). Hence, these results indicated that LDHA reduced the suc-CoA concentrations probably by inhibiting the levels and activity of α-KGDC.

Co-IP experiments showed that succinylated Txnrd1 levels were increased by upregulating suc-CoA concentrations (Fig. 8H). Rescue experiments showed that LDHA downregulated succinylated Txnrd1 protein levels and upregulated Txnrd1 protein levels, which could be abrogated by exogenous suc-CoA supplementation, suggesting that LDHA regulated Txnrd1 succinylation via suc-CoA (Fig. 8I). Using the succinylation site prediction online server SuccinSite [23] (http://syntbio.cau.edu.cn/SuccinSite/index.php), we found that K119, K195, K534 and K544 are potential succinylation sites on Txnrd1 (Fig. S14A). We subsequently mutated Txnrd1 residues K119, K195, K534 and K544 into Arg (R) and found that only Txnrd1 K534R significantly decreased suc-CoA-dependent Txnrd1 succinylation, indicating that K534 is the Txnrd1 succinylation site (Fig. 8J). The K534 site of Txnrd1 is conserved across various mammalian species (Fig. 8K). We then blocked Txnrd1 succinylation via mutation of Txnrd1 succinylation site K534 to investigate whether Txnrd1 ubiquitination was directly inhibited by Txnrd1 succinylation. Co-IP experiments showed that Txnrd1 ubiquitination was reduced after blocking Txnrd1 succinylation and this effect was abrogated by suc-CoA supplementation (Fig. 8L). Moreover, mutation of the Txnrd1 succinylation site K534 extended the half-life of Txnrd1 (Fig. 8M). Therefore, LDHA overexpression increased Txnrd1 protein levels by reducing Txnrd1 succinylation, which subsequently inhibited Txnrd1 ubiquitination.

3.9. LDHA-driven lactate production induced M2 macrophage polarization

The metabolomics data and basic measurement of lactate levels showed that LDHA overexpression increased intracellular lactate concentration by approximately 1.6-fold (Fig. 8D and S15A). We next evaluated whether LDHA overexpression in CMs could lead to acidosis since excessive lactate production could cause acidosis, followed by intracellular Na\(^+\) and Ca\(^{2+}\) overload (Fig. S15B). We found that LDHA overexpression had no significant effect on the intracellular pH, NHE1 and NCX1 proteins levels and Ca\(^{2+}\) concentrations in P7 CMs (Figs. S15C–E), suggesting that moderate elevation of lactate concentration after LDHA overexpression would not cause CM acidosis and Na\(^+\) and Ca\(^{2+}\) overload. Meanwhile, LDHA overexpression upregulated lactate transporter MCT4 protein levels and extracellular lactate concentrations (Figs. S15F–G), indicating that CMs could avoid excessive accumulation of lactate after LDHA overexpression by reactively increasing the secretion of lactate.

We next investigated whether cardiomyocyte LDHA could regulate macrophage polarization. Western blotting and flow cytometry showed that LDHA overexpression promoted macrophage M2 polarization in the adult M1 model (Fig. 9A–B). Besides, LDHA deficiency upregulated the M1-related markers and downregulated the M2-related markers in the neonatal AR model (Fig. S16A). LDHA deficiency reduced M2 macrophages and increased M1 macrophages, which was counteracted by sodium lactate supplementation in the neonatal AR model (Fig. 9C). Moreover, sodium lactate supplementation abrogated the downregulated proliferative rate induced by LDHA deficiency in the neonatal AR model (Fig. 9D). Collectively, these results revealed that LDHA-driven lactate production created a beneficial cardiac regenerative microenvironment by inducing M2 macrophage polarization.

4. Discussion

In this study, we investigated whether LDHA promoted CM proliferation by inducing metabolic programming and found that LDHA overexpression promoted CM proliferation and enhanced cardiac functional recovery after injury by reducing suc-CoA levels and increasing lactate levels. In details, LDHA-mediated suc-CoA reduction inhibited succinylation-dependent ubiquitination of Txnrd1, which reduced ROS and thereby promoted CM proliferation. LDHA also increased lactate production moderately to create a beneficial cardiac regenerative microenvironment by inducing M2 macrophage polarization. Our study
Fig. 4. LDHA overexpression improved cardiac function after MI in adult mice. (A) Schematic of the MI experiments in adult mouse hearts injected with AAV9. Echo, echocardiography. (B) Ki67 staining in adult mouse MI model hearts at 21 days post-MI (866 CMs from 12 images of 6 mice in cTnT-NC and 1003 CMs from 12 images of 6 mice in cTnT-LDHA groups). (C) pH3 staining in adult mouse MI model hearts at 21 days post-MI (1459 CMs from 24 images of 6 mice in cTnT-NC and 1825 CMs from 24 images of 6 mice in cTnT-LDHA groups). (D) Aurora B staining in adult mouse MI model hearts at 21 days post-MI (4239 CMs from 6 mice in cTnT-NC and 3504 CMs from 6 mice in cTnT-LDHA groups). (E) WGA staining of left ventricle heart sections from adult MI model hearts at 21 days post-MI (219 CMs from 15 images of 5 mice in cTnT-NC and 212 CMs from 15 images of 5 mice in cTnT-LDHA groups). (F) Masson straining of heart sections in adult mice at 49 days post-MI. n = 8 mice. (I) Cardiac function was analyzed using echocardiography at pre-MI and 7, 21 and 49 days post-MI. n = 10 mice. Bars = 20 μm in B-E and 1 mm in F. Statistical significance was calculated using an unpaired t-test in B-D, nonparametric Mann-Whitney U test in E, one-way ANOVA in F and two-way ANOVA in H; *P < 0.05.
Fig. 5. LDHA overexpression induced metabolic reprogramming in CMs. (A) Schematic diagram of glycolysis and the TCA cycle. (B) Heatmap showing 30 metabolites concentration in P7 CMs after LDHA overexpression. The metabolites marked in red are significantly changed. (C–D) The levels of β-F6P, DHAP, pyruvate, lactate, suc-CoA and succinate detected in P7 CMs after LDHA overexpression. n = 6 cell samples. (E) Volcano plot displaying the differentially expressed proteins in P7 CMs after LDHA overexpression. (F–G) GO and KEGG pathway enrichment analyzed the differentially expressed proteins after LDHA overexpression. (H) GSEA was performed to analyze clusters of genes that regulate the cell cycle. (I–K) Heatmap showing the differentially expressed proteins related to the cell cycle, glucose metabolic process and oxidative phosphorylation. Statistical significance was calculated using an unpaired t-test in C–D; *P < 0.05. FC indicated fold change. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
**Fig. 6.** LDHA regulated ROS levels and oxidative stress in CMs.

(A) ROS staining in P7 CMs after LDHA overexpression. 18 images of 6 mice per group. (B) p-ATM staining in P7 CMs after LDHA overexpression (173 CMs from 18 images of 6 mice in Adv-NC and 178 CMs from 18 images of 6 mice in Adv-LDHA groups). (C) ROS staining in adult hearts after LDHA overexpression. 18 images of 6 mice per group. (D) 8-OHG staining in adult hearts (137 CMs from 18 images of 6 mice in cTnT-NC and 164 CMs from 18 images of 6 mice in cTnT-LDHA groups). (E) ROS staining in adult MI hearts at 21 days after LDHA overexpression. 12 images of 6 mice per group. (F) 8-OHG staining in adult MI hearts at 21 days after LDHA overexpression (212 CMs from 18 images of 6 mice in cTnT-NC and 204 CMs from 18 images of 6 mice in cTnT-LDHA groups). (G) ROS staining in neonatal AR models after LDHA and MitoQ interference. 15 images of 5 mice per group. (H) pH3 staining in neonatal AR models after LDHA interference and MitoQ treatment (886 CMs from 15 images of 5 mice in Cre-empty + PBS, 1272 CMs from 15 images of 5 mice in Cre-empty + MitoQ, 1526 CMs from 15 images of 5 mice in Cre-LDHA + PBS and 1294 CMs from 15 images of 5 mice in Cre-LDHA + MitoQ groups). Bars = 20 μm in A, C, E and G-H, 2 μm (upper) and 20 μm (lower) in B, and 20 μm (left) and 5 μm (right) in D and F. Statistical significance was calculated using an unpaired *t*-test in A-F and one-way ANOVA in G-H; *P < 0.05.
Fig. 7. Txnrd1 was the downstream protein of LDHA that reduced ROS and promoted CM proliferation. (A) Heatmap showing the differentially expressed proteins related to ROS metabolic process. (B) G6pdx, Txnrd1, Mfn2, Coq7, Ndufs4, Ndufs3 and Hbb-b2 protein levels in P7 CMs after LDHA overexpression. n = 4 cell samples. (C) G6pdx, Txnrd1, Coq7, Ndufs4 and Hbb-b2 protein levels in mouse hearts at different ages. n = 4 mice. (D) ROS staining in P7 CMs after Txnrd1 overexpression. 10 images of 5 mice per group. (E) 8-OHG staining in P7 CMs after Txnrd1 overexpression (2247 CMs from 5 mice in Adv-NC and 1927 CMs from 5 mice in Adv-Txnrd1 groups). (F) Aurora B staining in P7 CMs after Txnrd1 overexpression (2247 CMs from 5 mice in Adv-NC and 1927 CMs from 5 mice in Adv-Txnrd1 groups). (G) ROS staining in adult hearts after Txnrd1 overexpression. 8 images of 4 mice per group. (H) 8-OHG staining in adult hearts after Txnrd1 overexpression (78 CMs from 8 images of 4 mice in Adv-NC and 79 CMs from 8 images of 4 mice in Adv-Txnrd1 groups). (I) Ki67 staining in adult hearts after Txnrd1 overexpression (649 CMs from 8 images of 4 mice in Adv-NC and 727 CMs from 8 images of 4 mice in Adv-Txnrd1 groups). Bars = 50 μm (left) and 10 μm (right) in D, 50 μm (left) and 5 μm (right) in E, and 20 μm in F–I. Statistical significance was calculated using an unpaired t-test in B, D–I and one-way ANOVA in C. *P < 0.05.
Y. Chen et al.
indicated that targeting metabolic reprogramming through LDHA might be an effective strategy to induce CM proliferation.

Our findings provided new insights into the role of LDHA in regulating CM proliferation by metabolically controlling ROS reduction and inducing M2 macrophage polarization. LDHA could be upregulated by several pro-cardiac regeneration transcription factors such as HIF1α and ErbB2, suggesting that LDHA might be involved in cardiac regeneration. We applied multiple strategies to evaluate the CM proliferative effects of LDHA, including immunostaining of cell cycle re-entry markers and time-lapse imaging. Additionally, we calculated CM regeneration. We applied multiple strategies to evaluate the CM proliferative effects of LDHA, including immunostaining of cell cycle re-entry markers and time-lapse imaging. Additionally, we calculated CM regeneration. We applied multiple strategies to evaluate the CM proliferative effects of LDHA, including immunostaining of cell cycle re-entry markers and time-lapse imaging. Additionally, we calculated CM regeneration. 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Fig. 9. LDHA-driven lactate production induced M2 macrophage polarization.
(A) Arg1, CD206, CD38, MMP9 and iNOS protein levels in adult MI model after LDHA overexpression. n = 6 mice.
(B) Flow cytometry analysis of macrophage polarization in adult MI model after LDHA overexpression. n = 5 mice.
(C) Flow cytometry analysis of macrophage polarization in neonatal AR models after LDHA and sodium lactate interference. n = 5 mice.
(D) Ki67 staining in neonatal AR models after LDHA interference and sodium lactate treatment (568 CMs from 15 images of 5 mice in Cre-empty + PBS, 590 CMs from 15 images of 5 mice in Cre-empty + Sodium lactate, 609 CMs from 15 images of 5 mice in Cre-LDHA + PBS and 592 CMs from 15 images of 5 mice in Cre-LDHA + Sodium lactate groups), bar = 20 μm (upper) and 10 μm (lower). Statistical significance was calculated using an unpaired t-test in A, two-way ANOVA in B and one-way ANOVA in C-D; *P < 0.05.
damage [44]. Other studies showed that half-molar sodium lactate infusion improved cardiac performance in patients with acute heart failure [45] and sodium lactate (2 g/kg/day) improved cardiac function after MI in mice [46]. These studies suggested that whether lactate production is beneficial or harmful to cardiac repair depends on lactate concentration. In our study, LDHA overexpression in P7 CMs increased the intracellular lactate concentration from 3.46 to 5.67 nmol/10^6 cells without significantly changing the intracellular pH and Ca^{2+} concentration. Hence, LDHA could be safely overexpressed in the heart for the treatment of MI.

The lactate dehydrogenases contain three genes LDHA, LDHB and LDHC [47]. Recent studies reported that LDHB and LDHC could regulate the metabolic remodeling of cancer cells and macrophage metabolism in the tumor microenvironment [48–50]. In our study, the proteomic data showed that LDHA expression was not affected after LDHA overexpression and the testis-specific gene LDHC [47] were not detected in CMs. We suspected that LDHA might be the mainly acting protein in our study. Despite of this, it still could not completely rule out some potential contributions of LDHB and LDHC in cardiac regeneration. Hence, it would be interesting to evaluate whether LDHB and LDHC were involved in cardiac regeneration.

This study has several limitations. Firstly, since multiple mechanisms that may be playing a role in LDHA-mediated CM proliferation based on the data of proteins and metabolites, we should conduct safety assessments in clinical translational research when considering LDHA as a therapeutic target for CM proliferation. Secondly, although our results indicated that LDHA overexpression could regulate the p-ATM and CDK1 expression and induce M2 macrophage polarization, we did not show clearly how LDHA-mediated CM proliferation. It is worthy investigating whether LDHA-mediated ROS alleviation promoted CM proliferation via the p-ATM/Wee1/CDK1 axis and whether lactate-induced M2 macrophage polarization promoted CM proliferation via cytokines such as IL-4 and IL-6 in future studies. Thirdly, our proteomics data showed that LDHA overexpression upregulated the extracellular matrix and fibroblast-specific proteins Lamb3, Coll5a2 and Coll1a2. Extracellular matrix proteins such as SLIT2 and NPNT were reported to regulate CM cytokinesis by mediating the crosstalk between CMs and CFs [51]. Besides, Collagen V deficiency could increase the scar size post-MI [52]. Hence, whether Lamb3, Coll5a2 and Coll1a2 are involved in the crosstalk between CMs and CFs is worthy of exploration. Furthermore, we only evaluated the role of LDHA in adult male mice, whether females still suffer from the adverse effects of MI and have different mechanisms mitigate cardiac injury and mediate cardiac repair is worthy of examination in future studies. Last but not least, since the hemocytometer cell counting method is dependent on the success of the initial cardiomyocyte isolation, we should pay attention to the successful rate and intra-group balance of this method to avoid false positive results.

5. Conclusions

In summary, LDHA-mediated metabolic reprogramming promoted CM proliferation by alleviating ROS and inducing M2 macrophage polarization. Our study suggested a new direction for promoting CM proliferation by targeting metabolic reprogramming.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102446.

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