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RESEARCH LETTER

Glutaminolysis is Essential for Myofibroblast Persistence and In Vivo Targeting Reverses Fibrosis and Cardiac Dysfunction in Heart Failure

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After injury, cardiac fibroblasts (CFs) differentiate into highly specialized myofibroblasts, essential for maintaining myocardial structural integrity by contracting surrounding tissue and remodeling the extracellular matrix network.[1] It was recently reported that withdrawal of stress stimuli is sufficient for some myofibroblasts to revert to a quiescent phenotype,[2] suggesting the potential for myofibroblast reversion by targeting the molecular pathways maintaining the fibrotic phenotype. Metabolic reprogramming has become appreciated as a primary trigger of cell fate transition, directing metabolic intermediates into pathways necessary for bioenergetics, anabolism, and the synthesis of cofactors regulating epigenetic modifiers and downstream gene expression. We recently reported that glutaminolysis (glutamine→glutamate) was linked to histone demethylation mediating myofibroblast formation,[3] implicating metabolic remodeling as a critical feature of the differentiation program. However, the greatest therapeutic potential of targeting the myofibroblast phenotype lies in reversing fibrosis by dedifferentiating activated myofibroblasts after injury under chronic stress stimuli.

To determine whether glutaminolysis inhibition is sufficient to revert myofibroblasts to a quiescent phenotype and reverse tissue fibrosis, we targeted *Gls1* (glutaminase-1), the replace with committed step in of glutaminolysis, following fibroblast activation in a murine model of pressure-overload heart failure (HF). *Gls1*$^{fl/fl}$ mice[4] were crossed with a *Periostin* inducible-Cre mouse line, restricting Cre recombination to activated fibroblasts (*Postn*^{iCre}.)[2] Because periostin is only expressed after fibroblast activation and the in vivo half-life of GLS1 is ~5.1 days,[5] this strategy allows for deletion of *Gls1* exclusively after myofibroblast differentiation. Given the difficulty in accurately identifying fibroblasts by surface antigens, *Gls1*$^{fl/fl}$×*Postn*^{iCre} mice were subsequently crossed to a Cre-dependent reporter mouse line (tdTomato) to permit isolation and assessment of any activated fibroblasts previously or currently expressing periostin (Figure, A). Mice were fed tamoxifen chow (tamox; 40 mg/kg/d) 1 day after transverse aortic constriction (TAC) and for the remainder of the study. Maintenance on tamox allowed for tdTomato labeling and *Gls1* deletion in all CFs activated during pressure overload. Cardiac function was monitored before and every 2 weeks after TAC (Figure, B). All animal work was performed in accordance with Institutional Animal Care and Use Committee approval by Temple University and followed American Association for Accreditation of Laboratory Animal Care guidelines. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Western blot analysis of tdTomato+ CFs isolated by fluorescence-activated cell sorting confirmed *Gls1* deletion after TAC (Figure, C). Aortic pressures obtained 1 week after TAC indicated similar pressure gradients between control (*Postn*^{iCre}) and experimental (*Gls1*$^{fl/fl}$×*Postn*^{iCre}) mice (Figure, D). Determination of the number of CFs that had been activated after TAC, ~3% of all gated cells, revealed no significant difference between groups, confirming we

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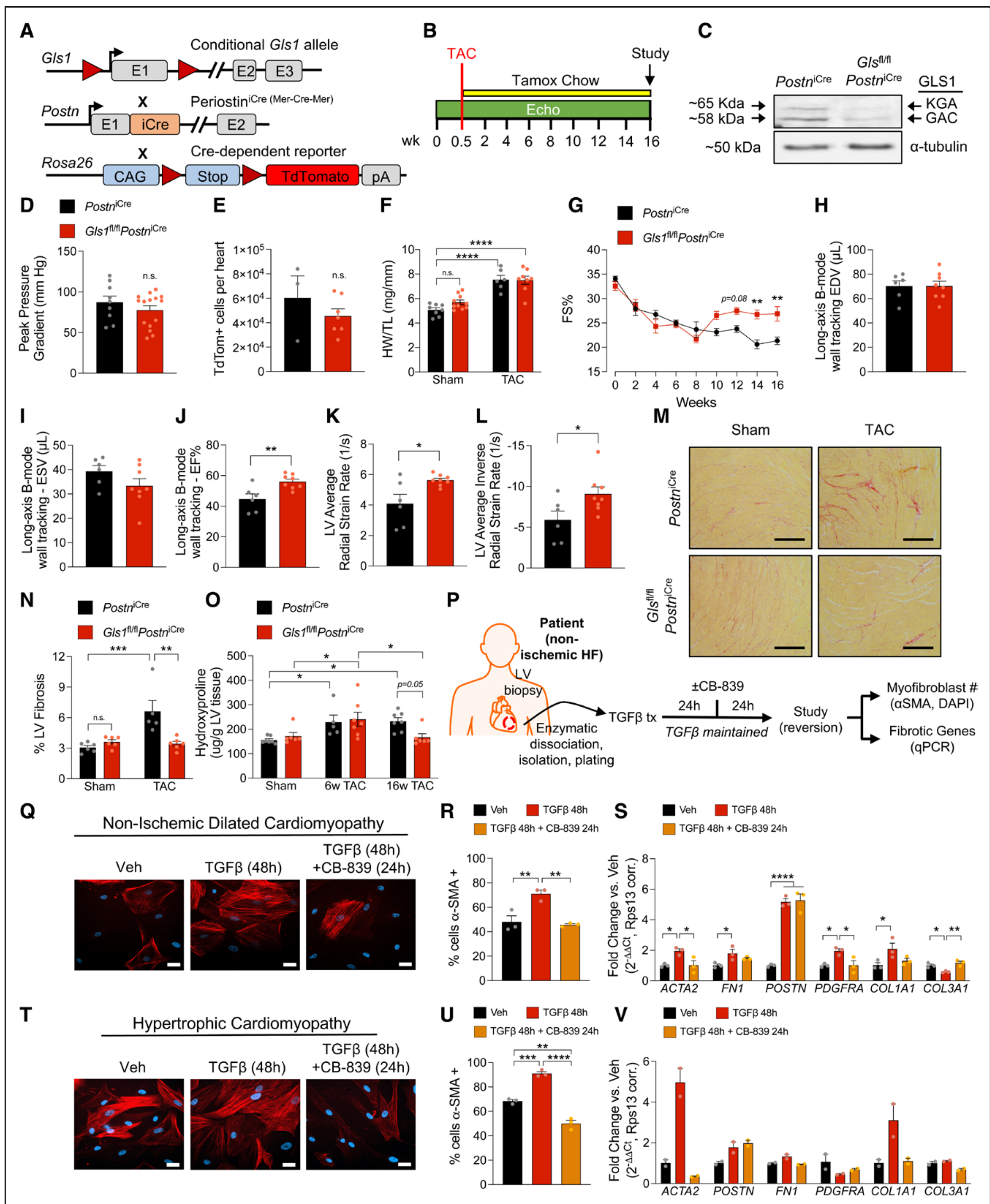

Figure. Inhibition of glutaminolysis in activated myofibroblasts reverses cardiac functional decline and fibrosis after murine pressure overload and reverts myofibroblasts isolated from nonischemic cardiomyopathy patients to a less fibrotic phenotype.
A, *Gls1*$^{fl/fl}$ mice were crossed with mice expressing a tamoxifen (tamox)–inducible, fibroblast-specific Cre recombinase (*Postn*^{iCre}), allowing for *Gls1* (glutaminase-1)–specific deletion in activated myofibroblasts, after differentiation. In addition, these mice were crossed to a Cre-dependent reporter line to allow for isolation and assessment of the activated and *Gls1*-deleted fibroblast population. **B**, Schematic of experimental timeline. Mice were fed tamox chow 1 day after transverse aortic constriction (TAC) and maintained on tamox chow for the duration of the study. Echocardiography was assessed at baseline and every 2 weeks after surgery. **C**, Western blot of tdTomato+ cells collected through fluorescence-activated cell sorting to confirm GLS1 deletion (KGA, full length; GAC, splice variant isoform). **D**, Aortic pressures 1 week after TAC. (*Continued*)

Figure Continued. **E**, Total TdTomato+ cells from hearts 16 weeks after TAC. **F**, Ratio of heart weight to tibia length (HW/TL) 16 weeks after TAC. **G**, Short-axis M-mode echo measurements of percent fractional shortening (FS%). Long-axis B-mode echo measurements of (**H**) end diastolic volume (EDV), (**I**) end systolic volume (ESV), and (**J**) percent ejection fraction (EF%) 16 weeks after TAC. **K**, Long-axis B-mode measurements of radial myocardial strain rates using speckle tracking echocardiography. **L**, Inverse strain rates indicative of the rate of myocardial relaxation. **M**, Representative images of hearts stained with picrosirius red to visualize and quantify tissue fibrosis after 16 weeks of TAC. Scale bar, 250 μm. **N**, Quantification of images in (**M**) as the percent of red stained area to total tissue area. **O**, Hydroxyproline content in left ventricular (LV) tissue samples. **P**, Schematic of cell acquisition, isolation, and experimental timeline to assess GLS1 inhibition on myofibroblast reversion under sustained stress stimuli (transforming growth factor–β [TGFβ]) in cardiac fibroblasts obtained from patients with nonischemic cardiomyopathy. **Q** and **T**, Representative images of cardiac fibroblasts chronically treated ± TGFβ for 48 hours. After the first 24-hour period, once myofibroblast formation had occurred, GLS1 inhibitor CB-839 (10 μM) was added and stained for α–smooth muscle actin (αSMA; red) and nuclear stain (DAPI; blue). Scale bar, 40 μm. **R** and **U**, Quantification of images in **Q** and **T** as the percent of cells expressing αSMA to the total number of cells (minimum of 80 cells quantified per replicate). **S** and **V**, Relative fold change in expression of myofibroblast genes (*ACTA2*, *FN1*, *POSTN*, *PDGFRA*, *COL1A1*, and *COL3A1*). *RPS13* was used as the loading control. Data shown as mean ± SEM, n=2 to 15 per group, as indicated. For statistical considerations, unpaired *t* tests (**D**, **E**, and **H**–**L**), 1-way analyses of variance with the Tukey post hoc test (**R**, **S**, **U**, and **V**), 2-way analyses of variance with the Tukey post hoc test (**F**, **N**, and **O**), or a 2-way mixed-effects model analysis of variance with Sidak post hoc test for multiple comparisons (**G**) was used (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). qPCR indicates quantitative polymerase chain reaction.

Nonstandard Abbreviations and Acronyms

CF	cardiac fibroblast
GLS1	glutaminase-1
HF	heart failure
TAC	transverse aortic constriction

were temporally deleting *Gls1* after fibroblast activation and differentiation (Figure, E). We did not observe any difference in cardiac hypertrophy (Figure, F), but we did observe preserved left ventricular fractional shortening from 10 to 16 weeks after TAC in *Gls1*$^{fl/fl}$×*Postn*^{iCre} mice (Figure, G). To further examine cardiac contractility and relaxation, B-mode speckle-tracking echocardiography was performed. Ejection fraction rebounded in *Gls1*$^{fl/fl}$×*Postn*^{iCre} mice 16 weeks after TAC (Figure, H–J). We also found left ventricular radial strain rates in *Gls1*$^{fl/fl}$×*Postn*^{iCre} mice to be significantly greater than in controls, indicating increased systolic function (Figure, K). We also saw enhanced inverse radial strain rates, a measure of the rate of left ventricular relaxation or diastolic function (Figure, L), an important finding because diastolic dysfunction highly correlates with tissue fibrosis. Assessment of collagen deposition (% fibrosis) revealed a significant increase in left ventricular fibrosis in control mice 16 weeks after TAC, which was prevented by targeting *Gls1* in activated myofibroblasts (Figure, M–O). It is important to note, collagen deposition was similar between groups at an earlier time point (6 weeks after TAC; Figure, O), confirming our hypothesis of fibrotic regression after loss of glutaminolysis in activated myofibroblasts. These in vivo results indicate the therapeutic potential of targeting glutaminolysis in activated myofibroblasts to reverse cardiac fibrosis and mitigate functional decline in HF.

To further determine the translational potential of targeting glutaminolysis for myofibroblast reversion, we isolated CFs from heart tissue of patients with nonischemic cardiomyopathy and maintained these cells under disease conditions (transforming growth factor–β stimulation for 48 hours), treating with a GLS1 inhibitor (CB-839; 10 μM) at the 24-hour time point (Figure, P). Research use of human heart tissues in this study was approved by the University of Pennsylvania institutional review board with written informed consent obtained from each of the heart transplant recipients providing explanted heart tissue for this research. Analysis of α–smooth muscle actin+ cells (% αSMA+, myofibroblast number) and activation state of the fibrotic gene program by quantitative polymerase chain reaction revealed pharmacologic inhibition of glutaminolysis in CFs isolated from patients with HF was sufficient to decrease myofibroblast number and revert the gene program to a less fibrotic phenotype (Figure, Q–V). These results provide direct evidence for therapeutically targeting glutaminolysis in patients with HF to positively alter the fibroblast population and curb active fibrosis.

Our results identify glutaminolysis as a critical mediator of myofibroblast persistence in a murine model of HF and in CFs isolated directly from failing human hearts. This could be attributable to the essential role of glutamine as a carbon source for bioenergetics, anabolism, collagen biosynthesis, and epigenetic regulation of gene expression, all of which contribute to myofibroblast differentiation and the fibrotic phenotype. Small molecule inhibition of GLS1 is in phase 2 cancer clinical trials, which if found to be safe, could be repurposed rapidly for the treatment of fibrotic disease.

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