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Myocardial regeneration in adriamycin cardiomyopathy by nuclear expression of GLP1 using ultrasound targeted microbubble destruction

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ABSTRACT

Recently GLP-1 was found to have cardioprotective effects independent of those attributable to tight glycemic control.

Methods and results: We employed ultrasound targeted microbubble destruction (UTMD) to deliver piggybac transposon plasmids encoding the GLP-1 gene with a nuclear localizing signal to rat hearts with adriamycin cardiomyopathy. After a single UTMD treatment, overexpression of transgenic GLP-1 was found in nuclei of rat heart cells with evidence that transfected cardiac cells had undergone proliferation. UTMD-GLP-1 gene therapy restored LV mass, fractional shortening index, and LV posterior wall diameter to nearly normal. Nuclear overexpression of GLP-1 by inducing phosphorylation of FoxO1-S256 and translocation of FoxO1 from the nucleus to the cytoplasm significantly inactivated FoxO1 and activated the expression of cyclin D1 in nuclei of cardiac muscle cells. Reversal of adriamycin cardiomyopathy appeared to be mediated by dedifferentiation and proliferation of nuclear FoxO1-positive cardiac muscle cells with evidence of embryonic stem cell markers (OCT4, Nanog, SOX2 and c-kit), cardiac early differentiation markers (NKX2.5 and ISL-1) and cellular proliferation markers (BrdU and PHH3) after UTMD with GLP-1 gene therapy.

Conclusions: Intranuclear myocardial delivery of the GLP-1gene can reverse established adriamycin cardiomyopathy by stimulating myocardial regeneration.

1. Introduction

There are nearly 5.7 million Americans with heart failure (HF) and approximately 670,000 new cases are diagnosed in the U.S. each year [1]. The ideal goal for HF therapy is myocardial regeneration, which has become a major goal of HF therapy since the discovery that cardiomyocytes are not terminally differentiated. There are various theories about the origin of regenerating cardiomyocytes, including self-replication of pre-existing adult cardiac muscle cells [2], differentiation of adult resident cardiac progenitor cells [3], dedifferentiation and proliferation of adult cardiac muscle cells [4] and transdifferentiation of fibroblast cells into cardiac muscle cells [5]. These findings have led to numerous clinical trials of stem cell therapy. However, a recent meta-analysis of 49 stem cell trials for HF identified several flaws and internal discrepancies and no detectable benefit in left ventricular ejection fraction [6]. It remains unclear whether myocardial regeneration in heart failure is able to reverse established cardiomyopathy.

This study proposed to evaluate the effect of gene therapy with glucagon-like peptide-1 (GLP-1) on adriamycin (ADM) induced cardiomyopathy in a rodent model. We chose to study GLP-1 because it has been found to have cardioprotective effects independent of those attributable to tight glycemic control [7]. Intravenous infusions of GLP-1 peptide to patients with myocardial infarction or chronic HF

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improved global LV function and the function of ischemic LV segments [8]. GLP-1 acts indirectly via GLP-1 receptors distributed on the membrane of cardiomyocytes. GLP-1R acts via cAMP generation to produce distinct downstream signaling events via intracellular calcium or ERK1/2 activation [7]. However, no data have been published regarding the effects of GLP-1 gene delivery to heart. The purpose of this study was to deliver GLP-1 gene directly to the hearts of normal rats or rats with HF induced by ADM. We employed ultrasound targeted microbubble destruction (UTMD) [9,10] which has been used to direct gene or protein therapy to specific organs in vivo and we chose to use a nuclear localizing signal to investigate the effects of GLP-1 gene delivered specifically to the nucleus using a piggybac transposon plasmid system [11,12] to rat hearts. After a single UTMD treatment, transgenic GLP-1 was overexpressed in nuclei of rat heart cells with evidence that transfected cardiac cells underwent dedifferentiation and proliferation.

2. Methods

2.1. Animal protocols and UTMD

All animal studies were performed in accordance with National Institute of Health (NIH) recommendations and the approval of our institutional animal research committee.

Animal protocol 1 was planned to test the ability of UTMD to deliver GLP1-NLS to the heart. A total of 18 normal rats received one of 3 treatments: (1) normal control rats, n = 6; (2) UTMD with pXL-BASII-CI-DsRed/pCI-hyPB (n = 6); (3) UTMD with pXL-BASII-CI-GLP1-NLS/pCI-hyPB (n = 6). Rats were euthanized at 4 weeks after UTMD. BrdU (100 mg/kg, Sigma, St Louis, MO) was injected intraperitoneally 6 h prior to euthanasia.

Animal protocol 2 was planned to evaluate the results of ADM on rat heart function and the concomitant administration of GLP1-NLS to attenuate the development of ADM-induced cardiomyopathy. A total of 60 rats were divided into five groups of 12 rats each: (1)normal control rats; (2) ADM injection only at total dose of 15 mg/kg/ip, 2.5 mg/kg/ip 6 times over 2 weeks [22]; (3) ADM plus human GLP-1 peptide (Sigma, St. Louis, MO), at a dose of 50 nmol/kg/ip/time × 6; (4) ADM injection plus UTMD-GLP1 peptide (same dose of human GLP-1 mixed with microbubbles for UTMD); (5) ADM injection plus UTMD with pXL-BASII-CI-GLP-1NLS/pCI-hyPB. One half of the rats were euthanized 16 h after the last ADM injection; the remaining rats were euthanized 4 weeks after UTMD.

Animal protocol 3 was planned to test the hypothesis that delivery of GLP1-NLS by UTMD could reverse established ADM cardiomyopathy defined as a fractional shortening <30% by echocardiography. ADM was first injected and rats with established cardiomyopathy were treated 2 weeks after ADM. There were 24 rats divided into two control groups of 6 rats each and a treatment group of 12 rats: (1) normal control rats; (2) UTMD with pXL-BASII-CI-DsRed/pCI-hyPB; (3) UTMD with pXL-BASII-CI-GLP-1NLS/pCI-hyPB. All rats were euthanized 4 weeks after UTMD.

2.2. RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated from 100 mg of harvested heart using the Trizol reagent (Invitrogen), according to the manufacturer's instructions and reverse-transcribed using Superscript III RT (Invitrogen). Quantitative RT-PCR analysis was performed on an ABI 7700 Sequence Detector (Applied Biosystems) using SYBR Green (RT2 SYBR Green qPCR Kit, Qiagen). Data were normalized to housekeeping gene expression (endogenous control). Changes in gene expression were normalized to control rat heart tissue. Complementary DNA PCR primer sequences information should be requested to the correspondence author.

2.3. Immunohistochemistry

Tissue samples were fixed 4% paraformaldehyde and 20% sucrose overnight at 4 °C for frozen sections. Cryostat sections 5–8 μm in thickness were further fixed with acetone (−20 °C) for 5 min. The slides that needed further nuclear protein retrieval were subjected to boiling citrate buffer solution pH 6.0 for 5–15 min. The primary antibodies were added and incubated for overnight at 4 °C. The secondary antibodies were added and incubated for 1 h at RT. BrdU staining included with incubating in HCl (1 N) for 10 min on ice to break open the DNA structure of the labeled cells and then followed by HCl (2 N) for 10 min at room temperature before moving them to an incubator for 20 min at 37 °C, Immediately after the acid washes, Borate buffer (0.1 M) is added to buffer the cells for 12 min.

2.4. Western blotting

Nuclear proteins extracts from cardiac tissue with a NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL 61105, USA). Equal amounts of protein were separated by SDS-PAGE. After separation and transfer to nitrocellulose membranes, the membranes were incubated with primary antibodies. Horseradish peroxidase secondary antibodies were used, and chemiluminescence was determined using the Super Signal West Dura detection system. All Western blots were performed in duplicate.

2.5. Plasmid construction

The piggybac transposon plasmid (pXL-BSII donor plasmid) was provided by Dr. Fraser MJ at University of Notre Dame (Notre Dame University, South Bend, IN) and hyperactive piggybac transposase helper plasmid was provided by Dr. Bradley A at Wellcome Trust Sanger Institute (Cambridge, UK). GLP-1 constructs: GLP-1 cDNA (7–37) with a furin cutting site after first ATG were constructed. We also fused a nuclear localization signal (NLS) fragment to GLP-1 cDNA. GLP-1NLS forward primers: 5-AAA-CTC-GAG-ATG-CGT-CAA-CGT-CGT-CAT-GCT-GAA-GGG-ACC-TTT-A-3; GLP-1NLS reverse primers: 5-AAA-AGC-CGC-TCA-GAC-CTT-CCG-CTT-TTT-TTT-AGG-TCC-TCG-GCC-TTT-CAC-CAG-CCA-3; DsRed cDNA vector was purchased from Clontech (Mountain view, CA), GLP-1NLS or DsRed cDNA with CMV promoter and polyA fragment with BgL II/BamH1 cutting subcloned to pXL-BSII vector.

2.6. Manufacture of plasmid-containing lipid-stabilized microbubble

Lipid-stabilized microbubbles were prepared as previously described in our laboratory [23]. Briefly, a solution of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (Sigma, St. Louis, MO) 2.5 mg/ml, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine 0.5 mg/ml, and 10% glycerol was mixed with 2 mg of pXL-BSII-CI-GLP-1 and pCI-hyPB (ratio is 5:1) dissolved in 50 μl of lipofectamine 2000 (Invitrogen, Carlsbad, CA). Aliquots of 0.5 ml of this phospholipid-plasmid solution were placed in 1.5 ml clear vials; the remaining headspace was filled with the perfluoropropane gas (Air Products, Inc, Allentown, PA). Each vial was incubated at 4 °C for 30 min and then mechanically shaken for 30 s by a dental amalgamator (VialmixTM, Bristol-Myers Squibb Medical Imaging, N. Billerica, MA).

2.7. Echocardiography

Echocardiographic measurements of LV mass, fractional shortening and LV posterior wall thickness were made from digital

images acquired with a 12 MHz broadband transducer (S12 probe, Philips Ultrasound, Bothell, WA) in M-mode under 2D echo short axis view.

2.8. Data analysis

Data was analyzed with Statview software (SAS, Cary, NC, USA). The results are expressed as mean ± SEM. Differences were analyzed by repeated measures ANOVA with Fisher's post hoc test and considered significant at $P < 0.05$.

3. Results

3.1. Overexpression of GLP-1 in nuclei of heart cells and reversal of ADM cardiomyopathy after UTMD-GLP-1NLS

Fig. 1 shows the absence of GLP-1 signal in the heart of normal rats (Fig. 1A) or UTMD-DsRed rats (Fig. 1B). However, GLP-1 is seen in the hearts of rats treated with UTMD-GLP1NLS (Fig. 1C), notably in nuclei of heart cells. GLP-1 was not detected in the bloodstream using GLP-1 ELISA kit (data not shown). Western blots was employed to detect GLP-1 from cardiac nuclear protein extracts and the results showed that GLP-1 signal existed in cardiac muscle nuclear protein extracts of UTMD-GLP1NLS (Fig. 1D). We further evaluated GLP1 mRNA level with quantitative RT-PCR. The result (Fig. 1E) shows that the GLP-1 mRNA level in UTMD-GLP1NLS was 84-fold greater than in normal or UTMD-DsRed ($P < 0.001$). We also counted the percentage of GLP-1-positive cells in heart slides. GLP-1 was present in 50.7% of cardiomyocytes, 16.7% of vascular endothelial cells, 14.3% of vascular muscle cells and 18.3% of vimentin-positive cells (Fig. 1F). We decided to evaluate if there is any possible pharmacological effects of GLP-1NLS on rat hearts with established adriamycin cardiomyopathy. Upper right panels of Fig. 1 shows the gross pathology of LV walls in the groups of ADM only, ADM plus GLP1 peptide, and ADM plus UTMD-GLP1 peptide (B, C, and D). Rats with ADM cardiomyopathy (B, C) had thin LV walls and dilated LV cavities compared to normal controls (A). ADM rats treated with UTMD-GLP-1NLS early (E) or 14 days later (F) resembled normal controls. The lower right panel shows the results of masson's trichrome staining for fibrosis, which was significantly decreased after UTMD-GLP1-NLS (E and F). Supplementary Fig. 2–3 demonstrated decreased LV mass and wall thickness in ADM cardiomyopathy with restoration to normal values by GLP-1NLS gene therapy but not GLP-1 peptide therapy. Similar findings were seen for LV fractional shortening and volume of abdominal ascites.

3.2. Nuclear GLP-1 inactivates FoxO1 by phosphorylation of FoxO1 at site of Ser-256, leads to translocation FoxO1 from the nucleus to the cytoplasm and activates myocardial overexpression of cyclin D1

Recently the translocation of FoxO proteins (forkhead box proteins, O) from the cytoplasm to the nucleus in cardiomyocytes was described as a key factor mediating the development of diabetic cardiomyopathy or ischemic cardiomyopathy. To our knowledge, this is the first report showing that FoxO1 is involved in ADM cardiomyopathy. Fig. 2C, E and F shows that FoxO1 signal is present in nuclei of cardiac muscle cells with established ADM cardiomyopathy. However, in normal rat heart slides we did not see FoxO1 signal in nuclei of cardiac muscle cells, although it was occasionally present in cytoplasm (Fig. 2A–B). After UTMD GLP-1-NLS gene delivery, transgenic nuclear overexpression of GLP-1 gene was associated with disappearance of nuclear FoxO1 and translocation of FoxO1 from nucleus to cytoplasm (Fig. 2D, H and I). The results (Fig. 2K) of FoxO1 western blots confirm a significant increase of

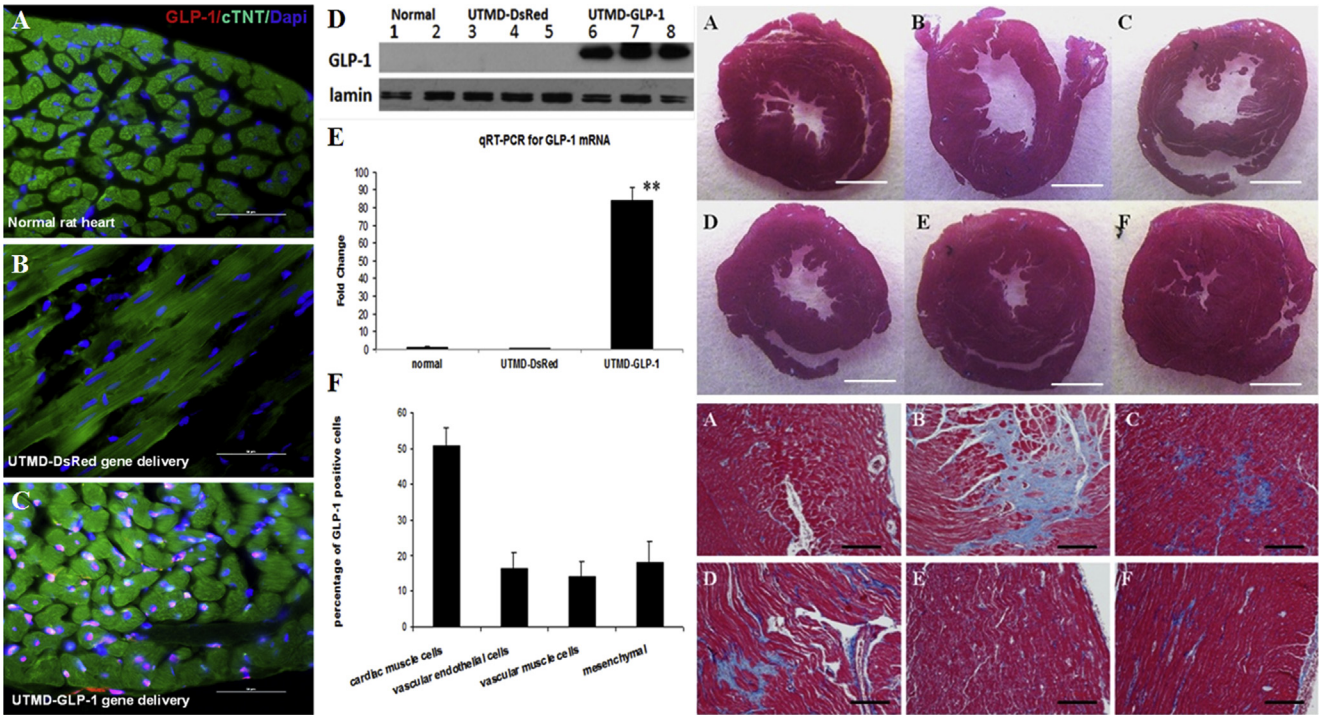

Fig. 1. Nuclear localization of GLP-1 signal in cardiac cells. (A–C) GLP-1/cTNT/Dapi staining. Scale bar is 50 μm. (D) western blots to detect GLP-1 from nuclear protein extract of heart tissue. (E) qRT-PCR for GLP1 cDNA. (F) Percentage of cell type in GLP-1-positive cells. Values are presented as mean ± SEM. $n = 6$ per group; **$P < 0.001$ *vs* control groups. Right panel is masson's trichrome staining. (A) Normal rat heart, (B) ADM only, (C) ADM plus GLP peptide, (D) ADM plus UTMD-GLP1 peptide, (E) ADM plus UTMD-GLP1NLS, (F) ADM injection first and 14 day late UTMD-GLP1NLS. The upper of right panel is Masson's trichrome staining for whole heart crossing section. Scale bar is 2.5 mm. The lower right panel is lower power imaging, scale bar is 100 μm.

FoxO1 in cardiac nuclear protein extracts of the ADM only group, and FoxO1 decreased to a nearly normal level after UTMD GLP-1NLS gene delivery. The result (Fig. 2L) shows that the FoxO1 mRNA level in the ADM only group was 12.6-fold greater than in normal control or normal plus UTMD-GLP-1NLS groups ($P < 0.001$). However, FoxO1 mRNA level was decreased to 4.5-fold ($P < 0.01$ vs ADM only) after UTMD GLP-1NLS gene delivery. The phosphorylation of FoxO1-S256 results in an inactivated form that is transferred from the nucleus to the cytoplasm, leads to loss of function of FoxO1. Supplementary Fig. 4 shown that FoxO1-phospho-S256 were detected in the nucleus and cytoplasm of cardiac muscle cells in normal rat heart (panels A, D, E and F), however, in the ADM only group the phosphorylation of FoxO1 were inhibited, leading to FoxO1 concentrating in the nuclei of cardiac muscle cells (panels B, G, H and I). Interestingly FoxO1-phospho-S256 was seen in the nucleus and the cytoplasm of cardiac muscle cells again after UTMD-GLP1NLS gene delivery (panels C, J, K and L). FoxO1-phospho-S256 western blots (panel M) confirm a significant

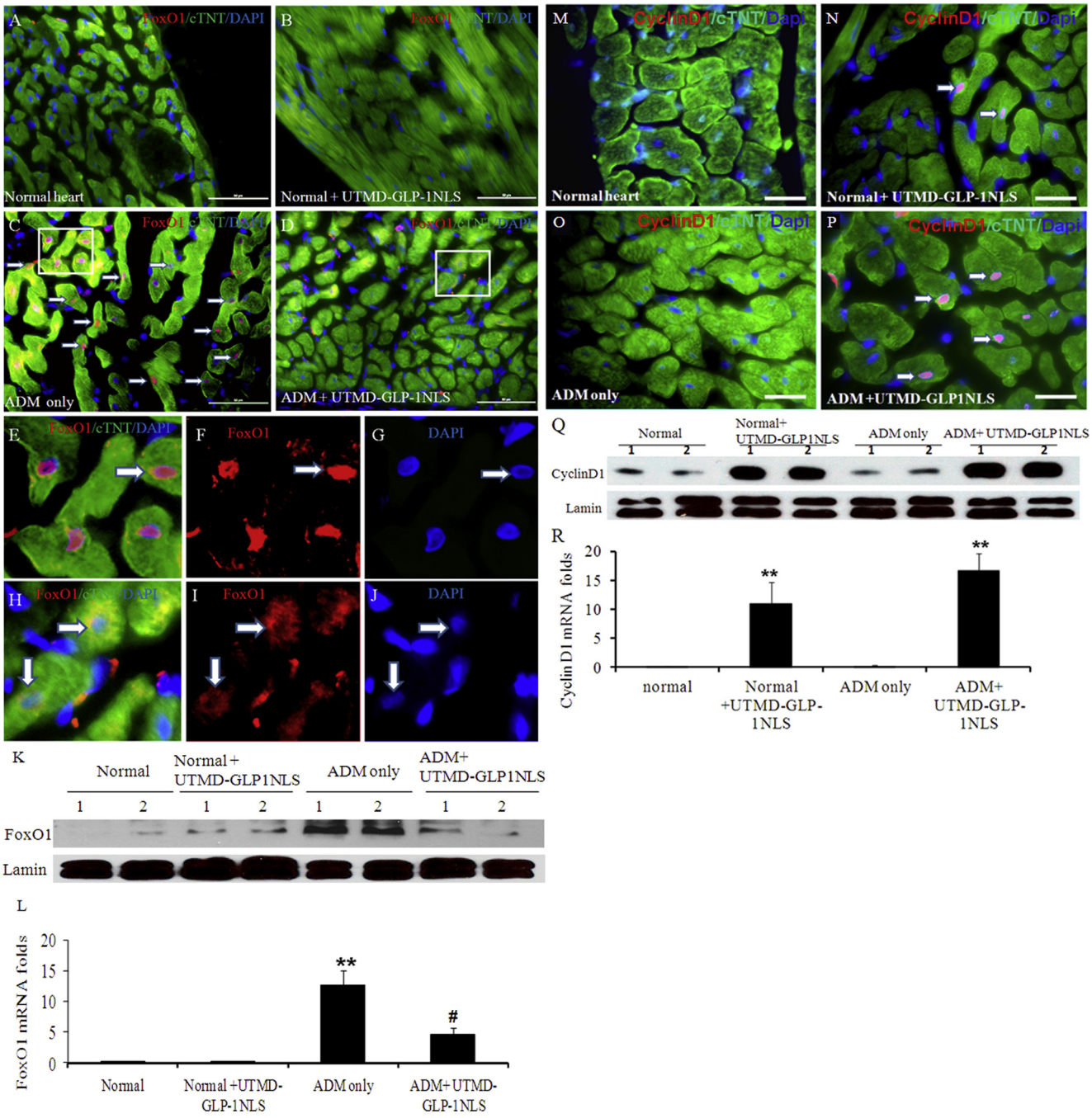

Fig. 2. Myocardial nuclear overexpression of FoxO1 induced by adriamycin. (A) Normal rat heart, (B) GLP1NLS gene delivered to normal rat heart, (C) ADM only, (D) ADM plus UTMD-GLP1NLS, (E–G) Further magnification from (C), (H–J) Further magnification from (D). Scale bar is 50 μm. (K) Western blots for detecting nuclear FoxO1 from nuclear protein extracts of heart tissue, (L) qRT-PCR for FoxO1 mRNA level, Values are presented as mean ± SEM, $n = 6$ per group; **$P < 0.001$ *vs* normal and normal plus UTMD-GLP-1NLS, #$P < 0.001$ *vs* ADM only. Right panel shows Overexpression of myocardial nuclear cyclin D1. (M) Normal rat heart, (N) Normal plus UTMD-GLP-1NLS, (O) ADM only, (P) ADM plus UTMD-GLP-1NLS, Scale bar is 25 μm. (Q) Western blots for detecting nuclear cyclin D1 from nuclear protein extracts of heart tissue, (R) qRT-PCR for cyclin D1 mRNA level, Values are presented as mean ± SEM. $n = 6$ per group; **$P < 0.001$ *vs* normal and ADM only.

decrease of FoxO1-phospho-S256 in cardiac nuclear protein extracts of the ADM only group, and FoxO1-phospho-S256 increased to a nearly normal level after UTMD GLP-1NLS gene delivery. The data taken together demonstrates that activation of myocardial nuclear FoxO1 mediates ADM cardiomyopathy and nuclear GLP-1 inactivates FoxO1 by the phosphorylation of FoxO1 at site of serine 256. CyclinD1does not exists in nuclei of adult cardiomyocytes of normal controls (Fig. 2M) and ADM only (Fig. 2O) but is seen in normal plus UTMD-GLP-1NLS (Fig. 2N) and ADM plus UTMD-GLP-1NLS (Fig. 2P). The result (Fig. 2Q) of cyclinD1 western blots also shows cyclinD1 expressed in cardiac nuclear protein extracts of normal plus UTMD-GLP-1NLS ADM plus UTMD-GLP-1NLS groups. The qRT-PCR (Fig. 2R) shows that the cyclinD1 mRNA level in Normal plus UTMD-GLP-1NLS and ADM plus UTMD-GLP-1NLS groups were 11.8-fold and 16.4-folds greater than in normal control or ADM only groups ($P < 0.001$).

3.3. Regenerating adult cardiomyocytes are in dedifferentiation

Although adult mammalian cardiomyocytes generally lose the capability of dedifferentiation and proliferation they exhibited in the fetus stage, we wondered if this capability could be recovered by genetic manipulation in ADM cardiomyopathy. Dedifferentiation of adult pancreatic beta cell [13] was mediated by the activation of nuclear FoxO1 under diabetic condition. Our data (Fig. 2) suggests that ADM induced overexpression of nuclear FoxO1. In addition, nuclear FoxO1-positive myocardium in ADM cardiomyopathy (Supplementary Fig. 5B, E and H) does not undergo dedifferentiation. However, after UTMD-GLP-1NLS, embryonic stem cell markers (OCT4, Nanog, and SOX2) were induced in nuclei of adult cardiac muscle cells (Supplementary Fig. 5C, F, and I). Further evidence to support dedifferentiation of adult rodent cardiomyocytes is co-expression of smooth muscle actin alpha (a marker of coronary artery muscle cells) with cardiac troponin T (a marker of mature cardiac muscle cell) (Supplementary Fig. 6F) in the sarcomere disassembly area. von Willebrand factor (vWf), a marker of vascular endothelial cells was not seen to co-localize with cTNT (Supplementary Fig. 6G, H and I). We also found that there are existed some c-kit positive adult cardiac muscle cells to support dedifferentiation of adult cardiac muscle cells into multipotent cardiac stem-like cells (Supplementary Fig. 7).

Fig. 3A–C shows the presence of NKX2.5-positive adult cardiomyocytes in adriamycin cardiomyopathy rats treated with UTMD-GLP1 gene therapy early and 14 day later but not in the controls. NKX2.5 signal was clearly seen in the nucleus of a small number of cardiac troponin T positive cells. Similar findings were

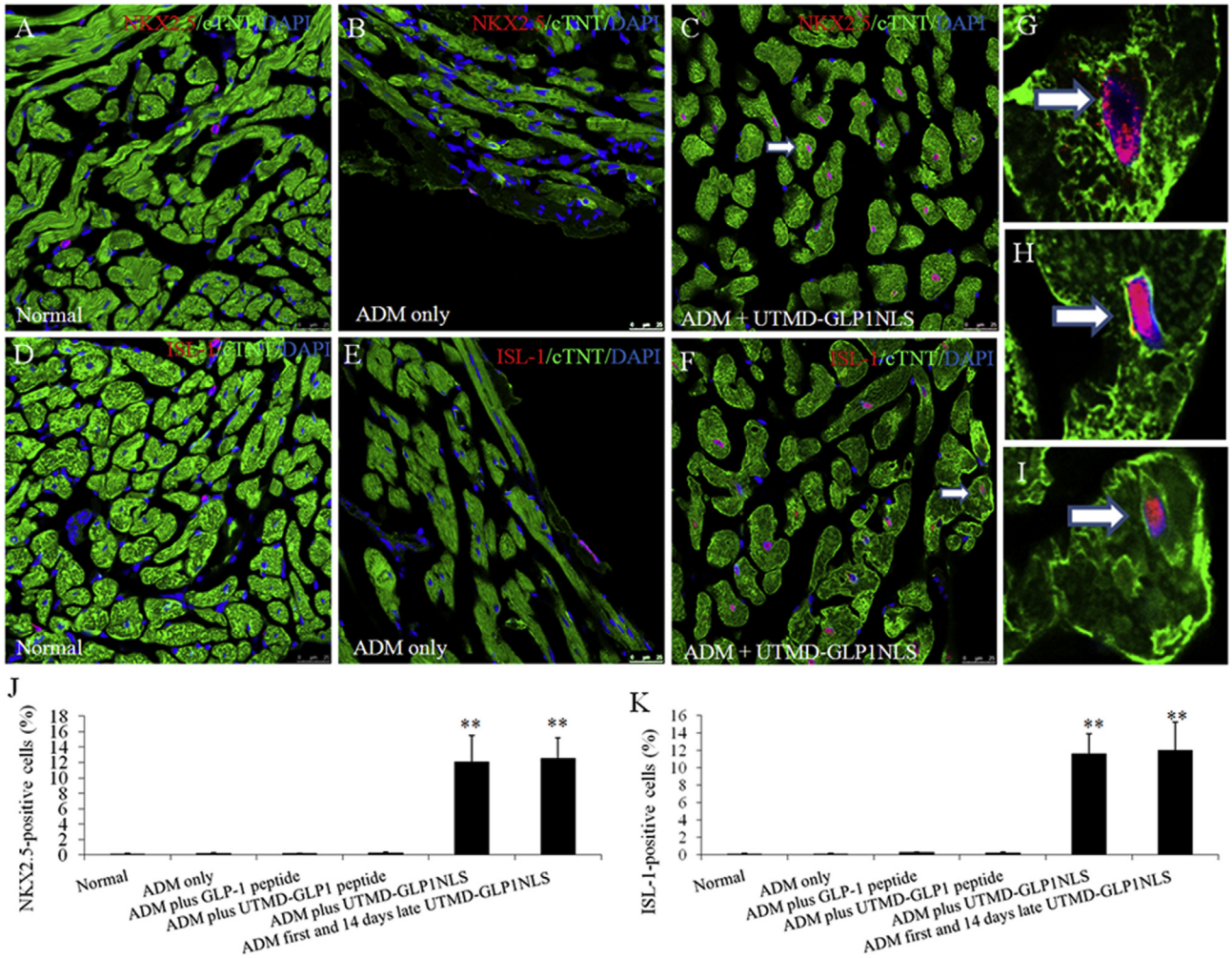

Fig. 3. Nkx2.5and ISL-1staining show that nuclear FoxO1-positive adult cardiomyocytes are differentiating. (A–C) Nkx2.5, (D–F) ISL-1, Scale bar is 25 μm. (G–I) Large arrowheads denote cardiomyocytes with disassembled sarcomeres, (J) a graph showing the percentage of NKX2.5-positive cardiac muscle cells. (K) a graph showing the percentage of ISL-1-positive cardiac muscle cells. Values are presented as mean ± SEM. $n = 6$ per group; **$P < 0.001$ *vs* control groups.

seen with ISL-1 (Fig. 3D–F). The percentage of NKX2.5 or ISL-1-positive cardiomyocytes was counted in 1000 cTnT positive cardiomyocytes cells in anterior wall and posterior LV walls with serial sections through each rat heart (n = 6 each group). The percentage of NKX2.5 positive cardiomyocytes in the gene therapy groups (Fig. 3J) was 8.73 ± 0.83% and 8.51 ± 1.45% ($p < 0.001$ vs controls). The percentage of ISL-1-positive cardiomyocytes (Fig. 3K) was 11.57 ± 2.31% and 11.97 ± 3.24% in the gene therapy groups ($p < 0.001$ vs controls). NKX2.5 and ISL-1 are considered markers of early cardiomyocyte differentiation [14]. Sarcomere disassembly is considered specific for cardiomyocyte proliferation [15]. We observed NKX2.5 and ISL-1-positive nuclei located in sarcomere disassembly structure (Fig. 3G, H and I). Thus, gene therapy with GLP-1NLS by UTMD appears to lead to dedifferentiation and proliferation of nuclear FoxO1-positive cardiomyocytes in the rat adramycin-induced cardiomyopathy model.

3.4. Dedifferentiated adult cardiomyocytes are in proliferation

We decided to use proliferation marker (anti-BrdU) and mitotic marker (anti-phospho-histone H3 (Ser10) (PHH3)) to demonstrate if dedifferentiated cardiac muscle cells were proliferating. We calculated the percentage of BrdU and PHH3 positive cardiomyocytes by counting stained nuclei (pink color (in web version)) from 1000 cTnT positive cardiomyocytes cells in the anterior and posterior LV walls using serial sections through each rat heart (n = 6 each group). Fig. 4C–D shows that BrdU signal was observed within the nucleus of cTnT positive cardiomyocytes by confocal microscopy in ADM plus UTMD-GLP-1NLS group. The percentage of BrdU positive cardiac muscle cells in the rats treated with UTMD-GLP1NLS gene therapy early and 14 day later (Fig. 4E) was 8.73 ± 0.83% and 8.51 ± 1.45% ($p < 0.001$ vs controls). Supplementary Fig. 8C–D show that PHH3 signal was observed within the nucleus of cTnT positive cardiomyocytes by confocal microscopy in ADM plus UTMD-GLP-1NLS group. The percentage of PHH3 positive cardiac muscle cells in the rats treated with UTMD-GLP1NLS gene therapy early and 14 day later (Supplementary Fig. 8E) was 7.73 ± 1.20% and 8.10 ± 1.70% ($p < 0.001$ vs controls).

4. Discussion

It was recently reported [16] that GLP-1r exists only in atrial, but not in ventricular cardiomyocytes, and that excited GLP-1r signal activates atrial ANP and mediates “so called GLP-1

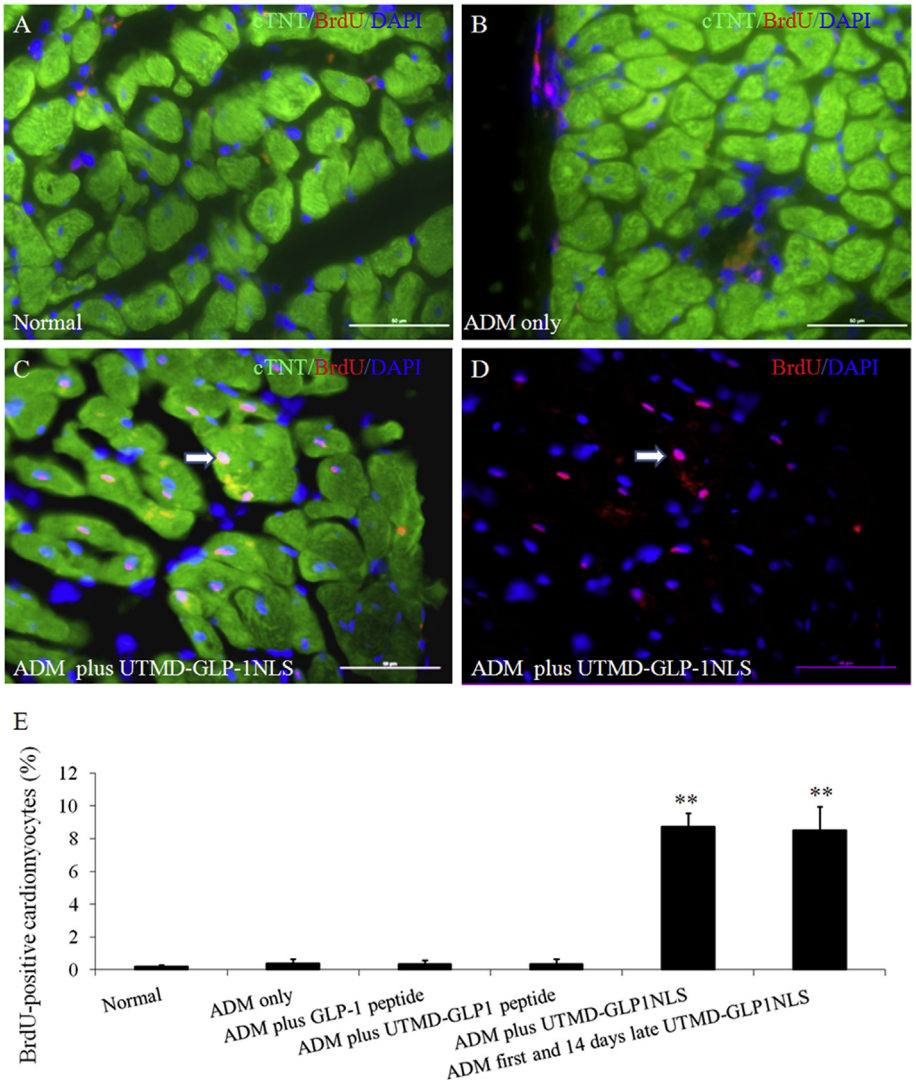

Fig. 4. BrdU staining shows proliferation in dedifferentiated adult cardiac muscle cells. (A) Normal group, (B) ADM only, (C) ADM plus UTMD-GLP1NLS, Scale bar is 50 μm. (E) a graphic for the percentage of BrdU-positive cardiomyocytes. Values are presented as mean ± SEM. $n = 6$ per group; **P < 0.001 *vs* control groups.

cardioprotection". Our data suggest that GLP-1 targeted to the cell nucleus of ventricular cardiomyocytes triggers sufficient myocardial regeneration to reverse established adriamycin cardiomyopathy in rats. While the exact mechanism whereby this occurs, remains to be completely elucidated, this is a novel finding that has important clinical implications for treatment of HF. Established adriamycin cardiomyopathy is a lethal disease. When HF develops, mortality is approximately 50% in a year. Moreover, treatment of established adriamycin cardiomyopathy is directly only to HF symptoms. There is no known therapy for reversing the underlying cardiomyopathy [17,18].

Battiprolu et al. [19] reported that metabolic stress–induced activation of FoxO1 in nuclei of cardiac muscle cells is central to the development of diabetic cardiomyopathy in diabetic mice, and the knockout FoxO1 blocked the formation of diabetic cardiomyopathy induced by a high fat diet. Our experiments shows that ADM cardiomyopathy is also associated with, and possibly mediated by, overexpression of FoxO1 in the nuclei of cardiac muscle cells. Interestingly, nuclear localization of GLP-1 in cardiac muscle cells appears to inhibit FoxO1 and restores nearly normal cardiac function and morphology after UTMD GLP-1 gene delivery. Abundant evidence now suggests that 3 members of the FoxO subfamily, FoxO1, FoxO3, and FoxO4, are critical for maintenance of cardiac function and cardiac stress responsiveness by effecting apoptosis and cell cycle regulation [20,21]. Nuclear FoxO1 controls cell cycle progression by down-regulation of cyclin D1 and apoptosis. So proliferative and antiapoptotic actions of GLP-1 require FoxO1 inactivation in ADM cardiomyopathy. Buteau J et al. [24] reported that GLP-1 caused a time-dependent translocation of FoxO1 from the nucleus to the cytoplasm, consistent with FoxO1 phosphorylation and induced FoxO1 inactivation in a pancreatic beta cell line. Our data clearly shows this phenomenon also occurs in ADM cardiomyopathy and that nuclear GLP-1 induced the phosphorylation of FoxO1 at the site of serine 256 and leads the translocation of FoxO1from the nucleus to the cytoplasm, removing the inhibition of nuclear FoxO1 on cyclin D1 that later mediates re-entry of cell cycle. This is the primary molecular mechanism of nuclear GLP-1 inducing myocardial proliferation or regeneration in ADM cardiomyopathy. Our data suggest that the process of myocardial regeneration is accompanied by dedifferentiation of nuclear FoxO1-positive adult cardiac muscle cells into cardiac progenitor-like cells expressing OCT4, Nanog, SOX2, c-kit, NKX2.5 and ISl-1 with subsequent redifferentiation into mature cardiac muscle cells. This phenomenon is not to known to occur in adult mammalian cardiac muscle cells under physiological conditions. Further investigation is needed to clarify if nuclear FoxO1-positive adult cardiomyocytes in cardiomyopathy are able to recover the capability of dedifferentiation and proliferation after intranuclear delivery of GLP-1 in nonhuman primates.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.038.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.038.

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