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Authors

Aminzadeh, Mohammad A
Tseliou, Eleni
Sun, Baiming
et al.

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BASIC SCIENCE

Therapeutic efficacy of cardiosphere-derived cells in a transgenic mouse model of non-ischaemic dilated cardiomyopathy

Mohammad A. Aminzadeh, Eleni Tseliou, Baiming Sun, Ke Cheng, Konstantinos Malliaras, Raj R. Makkar, and Eduardo Marbán*

Cedars-Sinai Heart Institute, 8700 Beverly Boulevard, Los Angeles, CA 90048, USA

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Aim Cardiosphere-derived cells (CDCs) produce regenerative effects in the post-infarct setting. However, it is unclear whether CDCs are beneficial in non-ischaemic dilated cardiomyopathy (DCM). We tested the effects of CDC transplantation in mice with cardiac-specific Gαq overexpression, which predictably develop progressive cardiac dilation and failure, with accelerated mortality.

Methods and results Wild-type mouse CDCs (10^5 cells) or vehicle only were injected intramyocardially in 6-, 8-, and 11-week-old Gαq mice. Cardiac function deteriorated in vehicle-treated mice over 3 months of follow-up, accompanied by oxidative stress, inflammation and adverse ventricular remodelling. In contrast, CDCs preserved cardiac function and volumes, improved survival, and promoted cardiomyogenesis while blunting Gαq-induced oxidative stress and inflammation in the heart. The mechanism of benefit is indirect, as long-term engraftment of transplanted cells is vanishingly low.

Conclusions Cardiosphere-derived cells reverse fundamental abnormalities in cell signalling, prevent adverse remodelling, and improve survival in a mouse model of DCM. The ability to impact favourably on disease progression in non-ischaemic heart failure heralds new potential therapeutic applications of CDCs.

Keywords Cardiomyopathy • Heart failure • Cell transplantation

Introduction

Cardiosphere-derived cells (CDCs) are regenerative and protective in the post-infarct setting, both in animal models[1–5] and in patients.[6] However, it is unclear whether CDCs exert any beneficial effects on non-ischaemic dilated cardiomyopathy (DCM). No models of DCM faithfully recapitulate the human condition in all its intricacies. Nevertheless, mice with cardiac-specific Gαq overexpression (Gαq mice) predictably develop progressive cardiac dilation and failure, and die prematurely in the absence of therapy.[7,8] The pathophysiology of Gαq overexpression involves oxidative stress, inflammation, and apoptosis[9–11] and thus mimics that of human heart failure in key respects.[12] Cardiosphere-derived cells are known to exert anti-inflammatory, anti-fibrotic, and anti-apoptotic effects on injured myocardium.[13] We, therefore, tested the concept that CDCs may be useful in a mouse model of DCM, by transplanting wild-type mouse CDCs into the hearts of Gαq mice. Cells were injected at various times during disease progression, so that we could detect either reversal of the phenotype, or prevention of further ventricular dysfunction.

Methods

Please see Supplementary material online, Methods. In brief, we studied male transgenic mice with cardiac-specific overexpression of Gαq [FVB/N-Tg (Myh6-Gnaq) 40Gwd/J] and male FVB/NJ mice (Jackson Laboratory, USA). Six-, 8-, and 11-week-old Gαq mice were randomized to CDC-treated or vehicle-treated groups and were followed for 3 months. FVB/NJ wild-type mice served as controls. Mouse CDCs were expanded from wild-type strain-matched

* Corresponding author. Tel: +1 3104237557, Fax: +1 3104237637, Email: Eduardo.Marban@cshs.org

mouse hearts (FVB/NJ wild-type mouse heart).[14] Intramyocardial injection, functional phenotyping, histological analysis, engraftment, and western blot protocols are described in Supplementary material online. Survival rate was assessed in Gαq mice treated with cells or vehicle at 8 weeks of age, using the Kaplan–Meier approach. To accelerate heart failure progression and mortality, adriamycin (5 mg/kg) was administered intraperitoneally twice (at a 5-day interval) 3 weeks after cell or vehicle treatment and once again 6 weeks after cell/vehicle treatment in Gαq mice and control FVB/NJ mice. Statistical analysis is described in detail in Supplementary material online. All the results are presented as means ± SEM. Analysis of variance followed by Bonferroni's *post hoc* test, Mann–Whitney *U* test, and Kruskal–Wallis test followed by Dunn's post test were applied in statistical analyses. The study followed pre-clinical reporting standards, as described previously.[15]

Results

Changes in global cardiac function

Dilated cardiomyopathy is characterized by progressive ventricular dilatation and contractile dysfunction. *Figure 1A* shows representative echocardiographic images of hearts from wild-type, vehicle-treated Gαq mice, and CDC-treated Gαq mice. Cardiosphere-derived cell transplantation resulted in a sustained improvement of LVEF (*Figure 1B–D*) and LV end-diastolic and end-systolic volumes (Supplementary material online, *Figure S1*) in Gαq mice, relative to placebo. This was true whether CDCs were injected at 6 (*B*), 8 (*C*), or 11 (*D*) weeks of age. Cardiosphere-derived cells did not restore lost function, but prevented the further progression of LV dysfunction

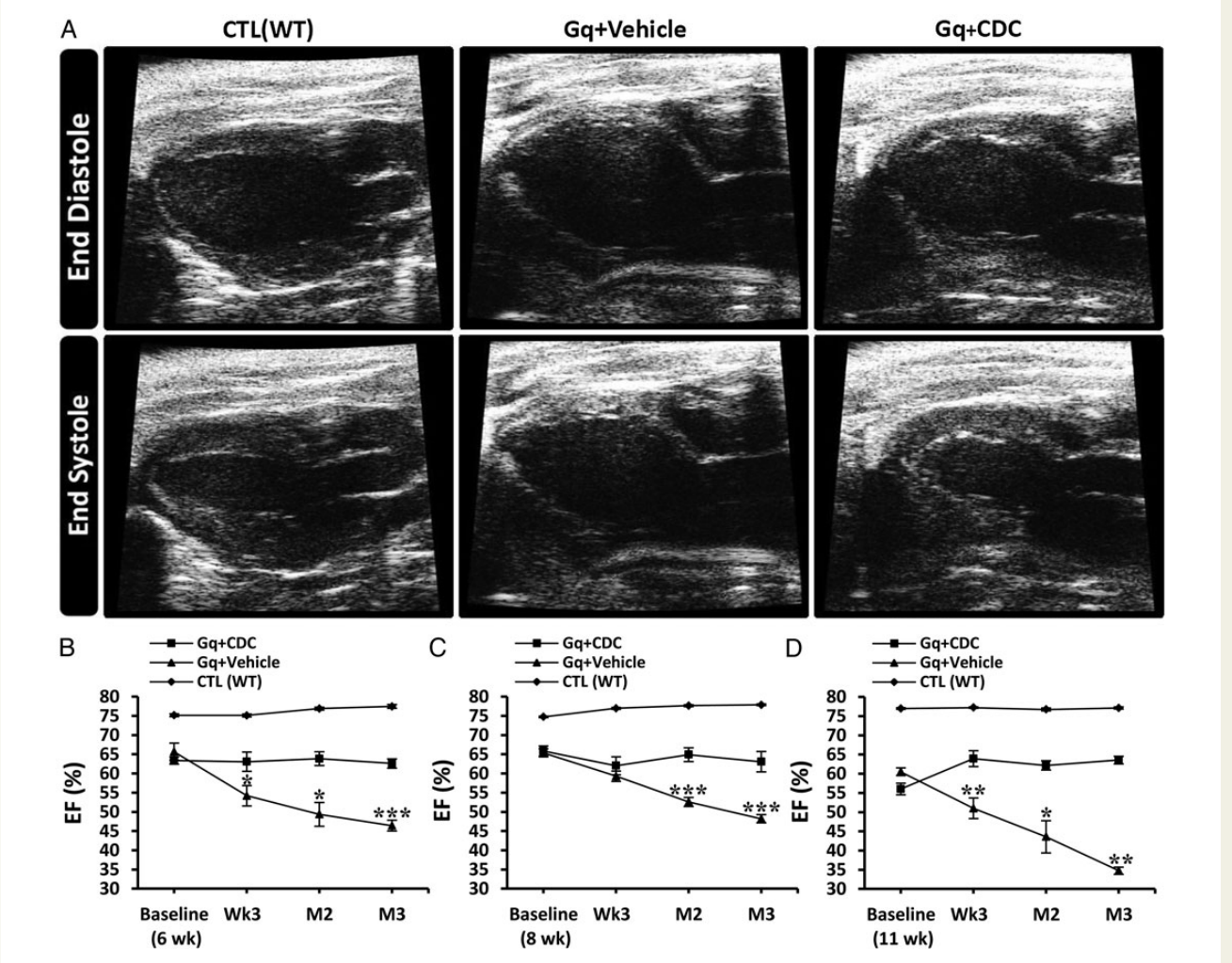

Figure 1 Functional benefits after cardiosphere-derived cell transplantation. (*A*) representative long-axis echocardiographic images at end-diastole (upper row) and end-systole (lower row) in control (CTL; wild-type) and in vehicle (Gαq+vehicle) and CDC (Gαq+CDC)-treated Gαq mice that were injected at 11 weeks of age at 3 months after treatment. Pooled data for left ventricular function show that cardiosphere-derived cell transplantation resulted in a sustained improvement of ejection fraction for 3 months in Gαq mice that received cardiosphere-derived cell at any of three different ages: 6 (*B*), 8 (*C*), and 11 (*D*) week old. Meanwhile, vehicle-treated Gαq mice showed significant deterioration of cardiac function. Data are means ± SEM; $n = 6–8$ in each group. The Mann–Whitney *U* test was applied. *$P < 0.05$ vs. Gq+CDC; **$P < 0.01$ vs. Gq+CDC; ***$P < 0.005$ vs. Gq+CDC.

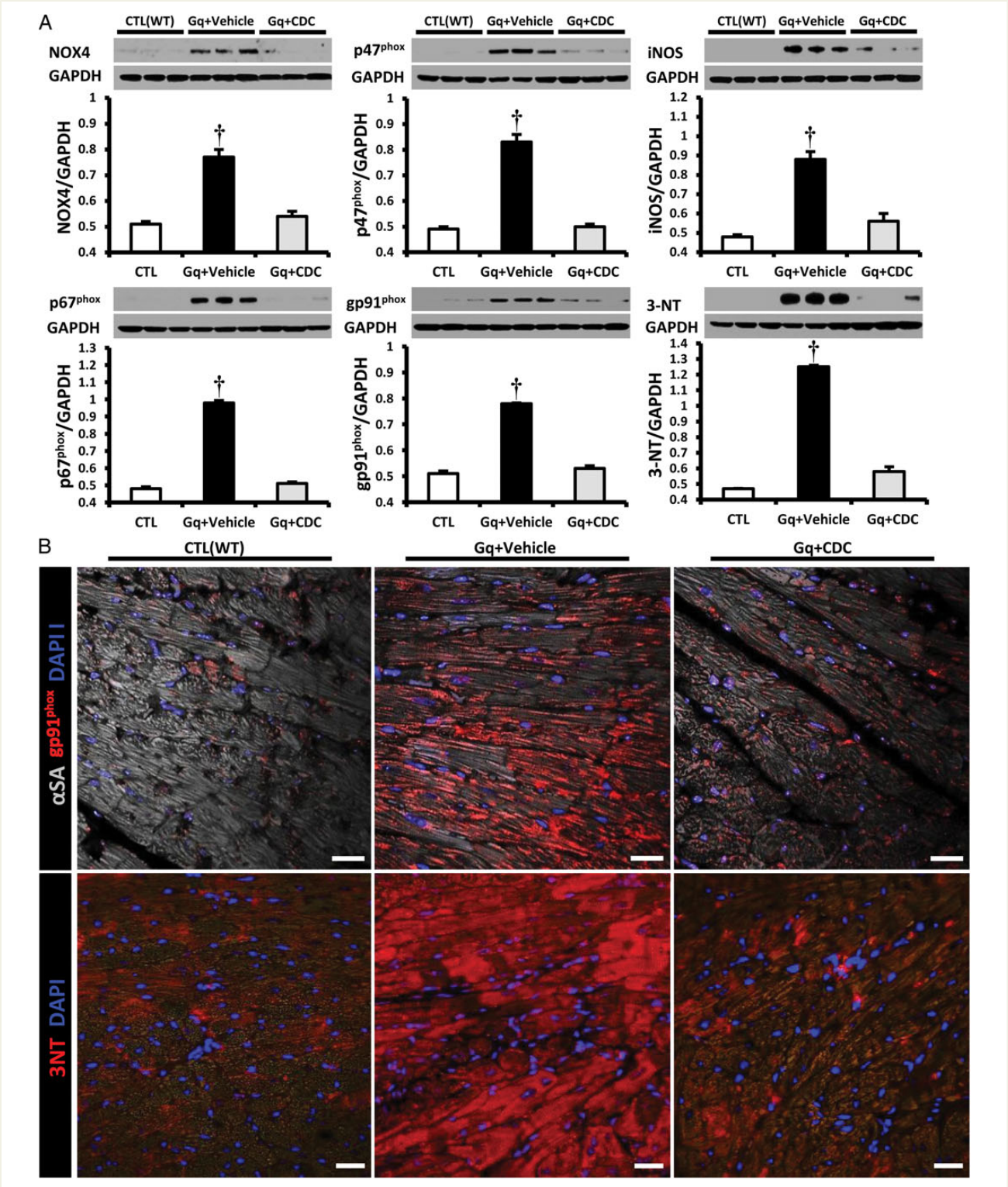

Figure 2 Prevention of oxidative/nitrosative stress with CDC treatment. Representative western blots and pooled data (*A*) and representative immunohistochemical images [*B*; CTL (wild-type), vehicle and cardiosphere-derived cell-treated Gαq mouse hearts stained for gp91^{phox}, and 3-nitrotyrosine] from Gαq mice treated at 8 weeks of age. Membranes were stripped and GAPDH probed as a control for loading in each lane. Data are means ± SEM; $n = 6–8$ in each group. $^{\dagger}P < 0.005$ vs. Gαq+CDC and control (CTL; wild-type). Scale bars: 20 μm.

which was evident in the vehicle-treated animals. Robust preservation of cardiac function was sustained in CDC-treated animals for at least 3 months. Cardiosphere-derived cells also resulted in sustained preservation of LV anterior and posterior wall thickness for at least 3 months, in contrast to placebo-treated animals (Supplementary material online, *Table S1* and *Figure S2*).

Cardiosphere-derived cell engraftment

Most of the benefit of CDC therapy is now recognized to be indirect, at least in ischaemic models.[13] Consistent with previous observations,[16] engraftment of CDCs at 1 week was ~10 and <1% at 3 weeks (Supplementary material online, *Figure S3*). By 6 weeks, no surviving CDCs could be detected. Thus, the long-term functional and structural benefits identified here must arise indirectly, rather than from direct differentiation of transplanted cells.

Oxidative stress and inflammation

Free radical injury is central to the pathophysiology of Gαq overexpression and DCM.[9–11] We sought evidence of oxidative and nitrosative stress by measuring the protein levels of various relevant enzymes (*Figure 2A*). In vehicle-treated Gαq mice, we found markedly increased protein abundance of the NOX4, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ subunits (shown also immunohistochemically in *Figure 2B*, upper row) of the major reactive oxygen species (ROS)-producing enzyme, NAD(P)H oxidase, and augmented protein density of inducible nitric oxide synthase (iNOS). These changes were associated with accumulation of 3-nitrotyrosine (*Figure 2A* and *B*, lower row), which results from NO reaction with ROS.

The oxidative/nitrosative stress was accompanied by intense inflammation [as manifested by increased abundance of monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), and nuclear factor kappa B p65 (NF-κB p65; *Figure 3A*), as well as accumulation of $CD68^+$ macrophages and $CD3^+$ T cells in hearts of vehicle-treated Gαq mice; *Figure 3B* and Supplementary material online, *Figure S4A*]. Thus, CDC treatment lowered 3-nitrotyrosine level, restored the protein abundance of NAD(P)H oxidase subunits, iNOS, MCP-1, and COX-2, prevented activation of NF-κB p65, reduced infiltration of inflammatory cells, and increased the number and percentage (Supplementary material online, *Figure S4*) of reparative anti-inflammatory macrophages (M2 subtype;$CD206^+$) in the myocardium of 8-week-CDC-treated Gαq mice. Additionally, *in vitro* assessment of CDC-conditioned media and its effect on cultured cardiomyocytes stressed with hydrogen peroxide, a strong oxidant, confirmed the antioxidative/anti-apoptotic effects of CDCs. The expression of cleaved caspase 3 was markedly lower in the stressed cardiomyocytes incubated with CDC-conditioned media compared with the cardiomyocytes incubated with cardiac fibroblast-conditioned media or basal media (Supplementary material online, *Figure S5*), providing further evidence for suppression of oxidant-induced apoptosis. We conclude that CDCs attenuate oxidative/nitrosative stress and inflammation in Gαq mice.

Protein kinase C–protein kinase D–cAMP response element-binding protein (remodelling) pathway and apoptosis

The PKC (protein kinase C)–PKD (protein kinase D)–CREB (cAMP response element-binding protein) pathway contributes to cardiac

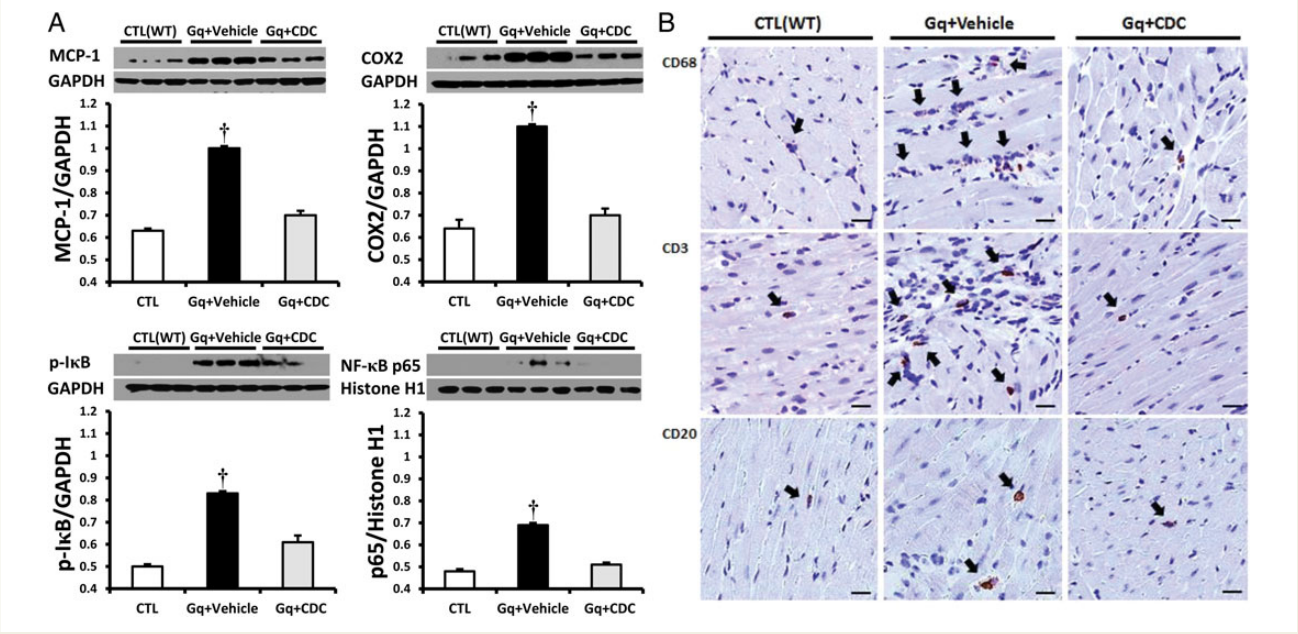

Figure 3 Suppression of inflammation with cardiosphere-derived cell treatment. Representative western blots and pooled data (*A*), and representative immunohistochemical images [*B*; CTL (wild-type), vehicle and cardiosphere-derived cell-treated Gαq mouse hearts stained for CD68, CD3, and CD20] from Gαq mice treated at 8 weeks of age. In CDC-treated Gαq mice, accumulation of $CD68^+$ macrophages (*B*, upper row) and $CD3^+$ T cells (*B*, middle row) was reduced. Membranes were stripped and GAPDH and histone H1 probed as a control for loading in each lane. Data are means ± SEM; $n = 6–8$ in each group. $^{\dagger}P < 0.005$ vs. Gαq+CDC and control (CTL; wild-type). Scale bars: 20 μm.

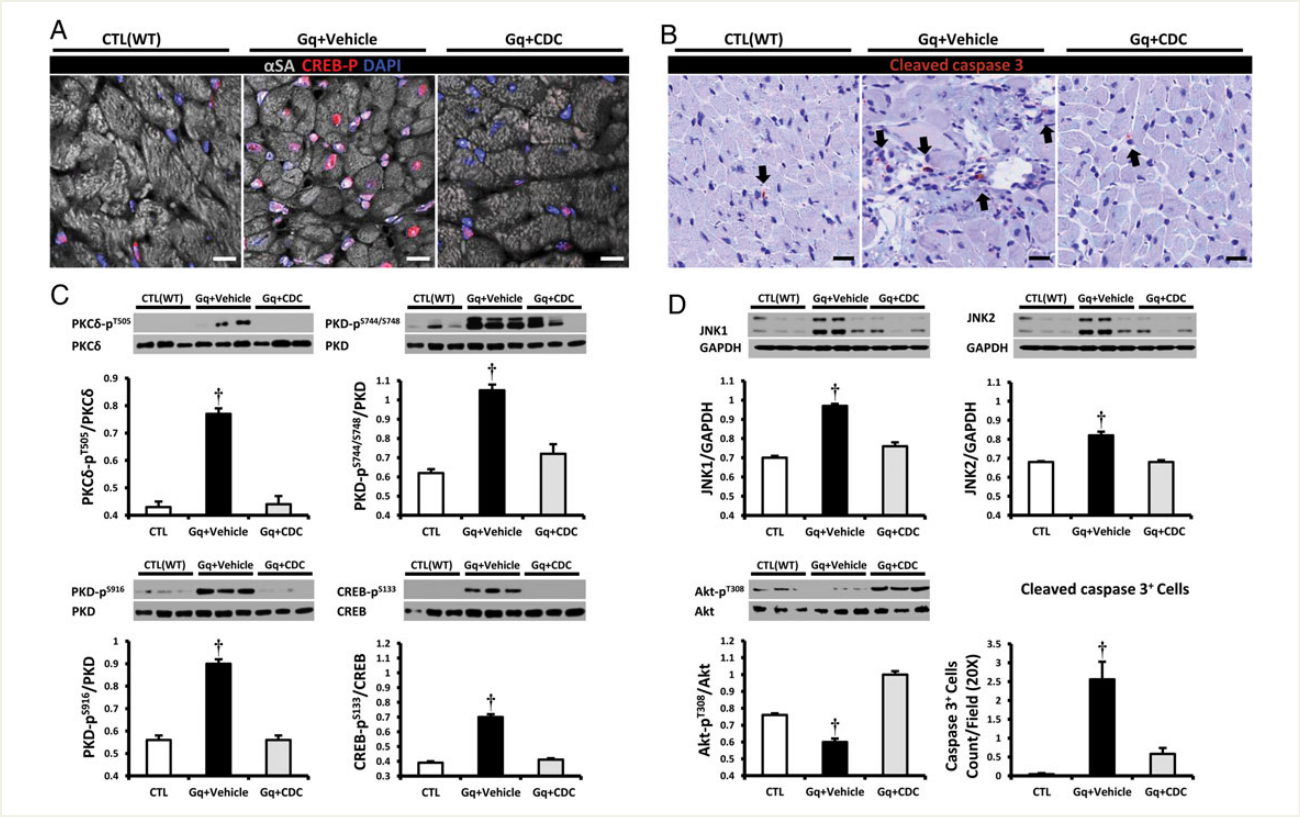

Figure 4 Restoration of the protein kinase C–protein kinase D–cAMP response element-binding protein remodelling pathway and suppression of apoptosis with cardiosphere-derived cell treatment. Representative immunohistochemical images [*A* and *B*; CTL (wild-type), vehicle and cardiosphere-derived cell-treated Gαq mouse hearts stained for p-cAMP response element-binding protein (Ser133) and cleaved caspase 3] and representative western blots and pooled data (*C* and *D*) from Gαq mice treated at 8 weeks of age. Cardiosphere-derived cell treatment restored the levels of relevant proteins in the protein kinase C–protein kinase D–cAMP response element-binding protein remodelling pathway to those in non-diseased controls (*C*). Increased protein density of PKCδ was associated with elevated nuclear contents of its downstream active effectors, phosphorylated protein kinase D (Ser744/748) and phosphorylated cAMP response element-binding protein (Ser133) in the vehicle-treated Gαq mice. (*B* and *D*) Increased abundance of active c-Jun N-terminal kinase, along with decreased active Akt protein density (Akt-p^{T308}) and markedly higher numbers of cells positive for cleaved caspase 3 in vehicle-treated Gαq mice. Activation of the protein kinase C–protein kinase D–cAMP response element-binding protein remodelling pathway and increased apoptosis were ameliorated by cardiosphere-derived cell therapy. Arrows in (*B*) point to apoptotic cleaved caspase 3^{+} cells. Membranes were stripped and GAPDH probed as a control for loading in each lane. Phosphorylated protein kinase C δ, protein kinase D, cAMP response element-binding protein, and Akt were normalized to total protein kinase Cδ, protein kinase D, cAMP response element-binding protein, and Akt, respectively. Quantification of JNK1 and JNK2 was performed using the same blots (D), taking advantage of the dual recognition of these two isoforms by the same antibody. Data are means ± SEM; $n = 6–8$ in each group. $^{\dagger}P < 0.005$ vs. Gαq+CDC and control (CTL; wild-type); scale bars: 10 μm (*A*), 20 μm (*B*).

remodelling in Gαq mice.[17] *Figure 4A* and *C* confirm that proteins involved in the PKC–PKD–CREB pathway are elevated in vehicle-treated Gαq mice, and that CDC treatment restored the levels of these proteins to those in non-diseased controls. Increased protein density of PKCδ was associated with elevated nuclear contents of its downstream active effectors, phosphorylated PKD (Ser744/748) and phosphorylated CREB (Ser133) in the vehicle-treated Gαq mice. We noted increased abundance of the pro-apoptotic mediator, active c-Jun N-terminal kinase (JNK), along with decreased active Akt protein density (Akt-p^{T308}) and markedly higher numbers of cells positive for cleaved caspase 3, a marker of apoptosis, in vehicle-treated Gαq mice (*Figure 4B* and *D*). Thus, the PKC–PKD–CREB remodelling pathway is activated in the myocardium of Gαq mice, with increased apoptosis, as described previously;[18] these pathophysiological signalling processes are ameliorated by CDC therapy.

Cardiac fibrosis

Cardiac fibrosis figures prominently in maladaptive responses to heart failure.[19] Augmentation of collagen production and angiotensin II-mediated secretion of Krüppel-like factor 5 (KLF5) are among the compensatory mechanisms mediated by cardiac fibroblasts in heart failure, but sustained activation exacerbates fibrosis and, ultimately, leads to decompensated heart failure.[20,21] In vehicle-treated Gαq mouse heart, collagen was increased [both histologically (*Figure 5A–C*) and at the protein level (*Figure 5D*)]; most of the collagen was co-localized with fibroblast-specific protein (FSP; *Figure 5B* and *C*). Cardiosphere-derived cell treatment markedly reduced cardiac collagen content, whether total or co-localized with FSP, and fibrosis (*Figure 5A–D*). In association with the increased abundance of collagen co-localized with FSP in vehicle-treated Gαq mouse heart, KLF5 and angiotensin II receptor type 1 (AT-R1)

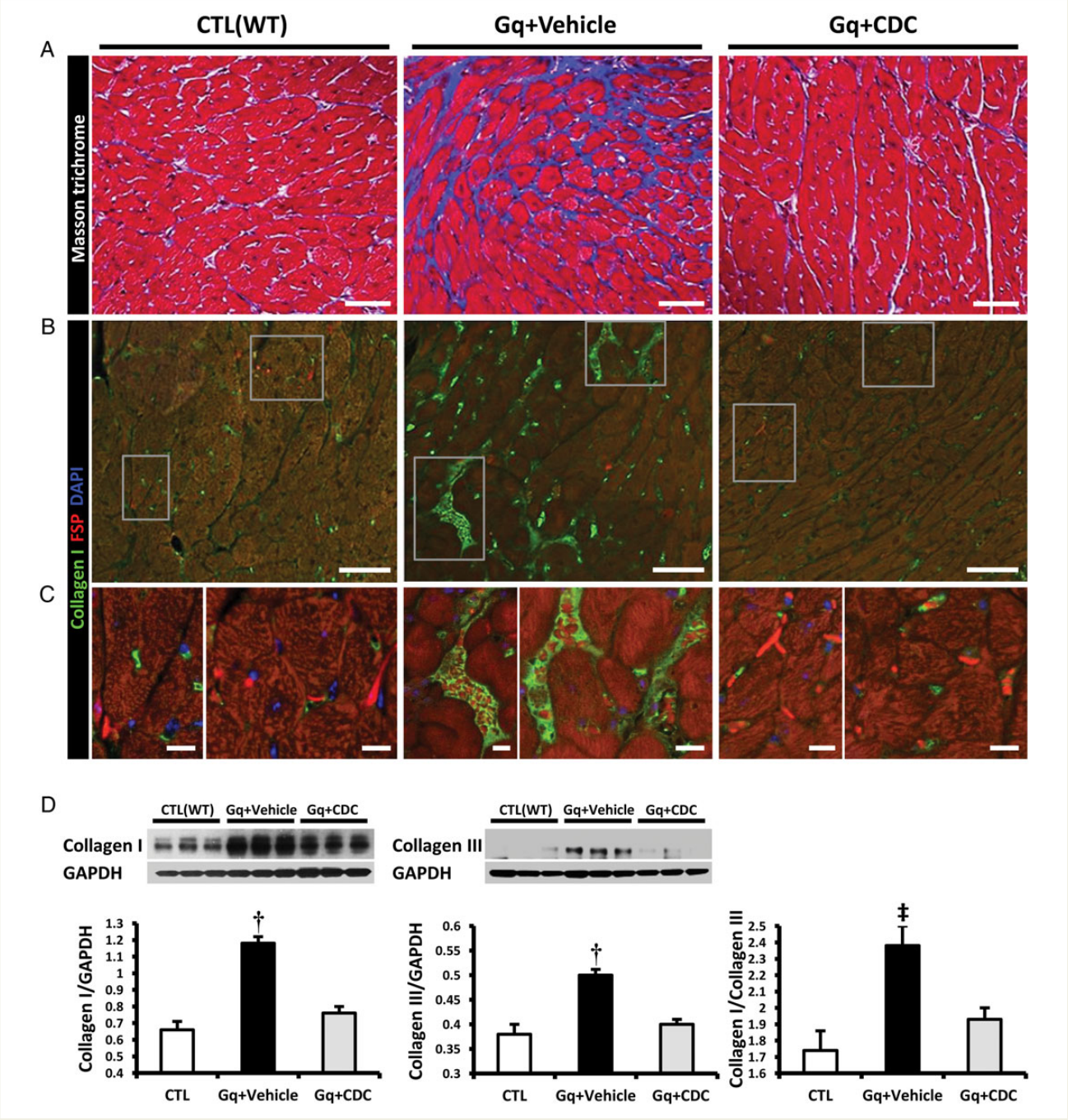

Figure 5 Cardiosphere-derived cell treatment markedly reduced cardiac collagen content and fibrosis. Representative Masson trichrome (*A*) and immunohistochemical images [*B* and *C*; CTL (wild-type), vehicle and cardiosphere-derived cell-treated Gαq mouse hearts stained for fibroblast-specific protein and collagen I] and representative western blots and pooled data (*D*) from Gαq mice treated at 8 weeks of age. Cardiosphere-derived cell treatment markedly reduced cardiac collagen content, whether total or co-localized with fibroblast-specific protein, and fibrosis (*A*–*D*). Membranes were stripped and GAPDH probed as a control for loading in each lane. Data are means ± SEM; $n = 6–8$ in each group. $^{\dagger}P < 0.005$ vs. Gαq+CDC and control (CTL; wild-type). $^{\ddagger}P < 0.05$ vs. Gαq+CDC and control (CTL; wild-type). Scale bars: 50 μm (*A* and *B*), 10 μm (*C*).

protein levels (*Figure 6A* and *B*) were augmented, indicating concomitant up-regulation of AT-R1 and KLF5 (presumably in cardiac fibroblasts). Cardiosphere-derived cell treatment negated the up-regulation of KLF5 and AT-R1. In addition to restoration of KLF5 and AT-R1 protein expression, the activity of matrix metalloproteinase 9 (MMP9) was markedly increased in the CDC-treated hearts 1 day after treatment compared with the vehicle-treated group (Supplementary material online, *Figure S6*). Matrix metalloproteinases play a critical role in collagen degradation and in remodelling of extracellular matrix proteins in the heart.[22] In

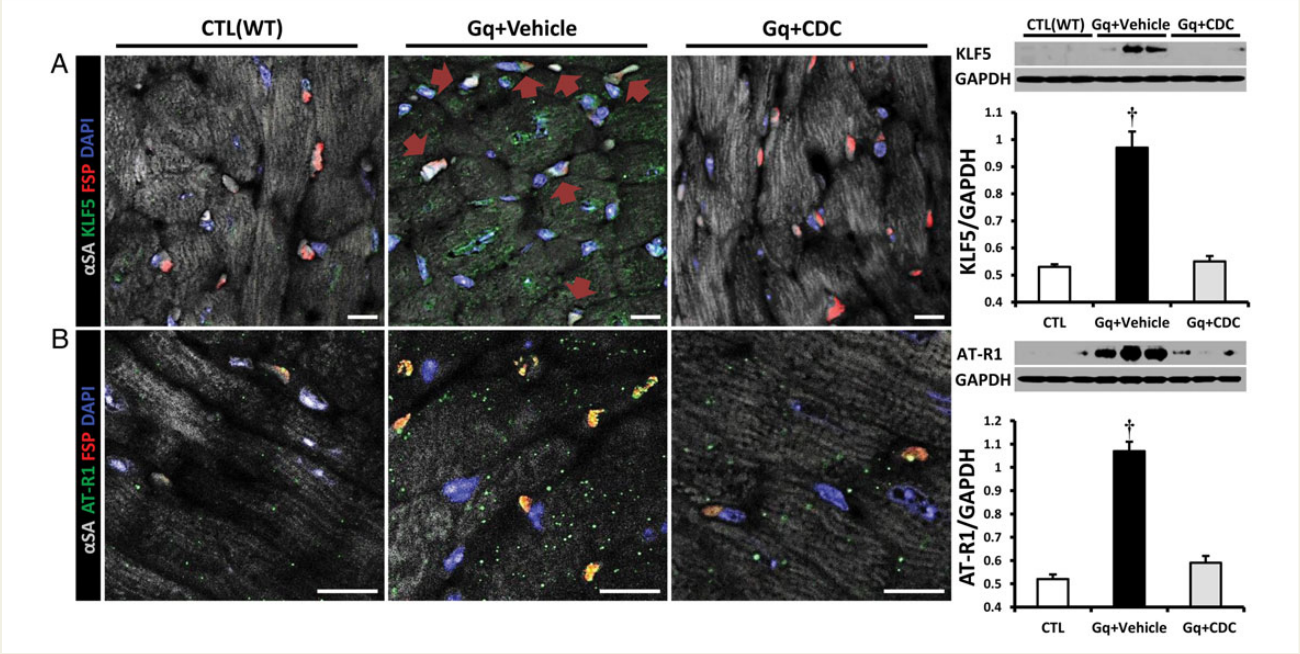

Figure 6 Cardiosphere-derived cell treatment restored expression of Krüppel-like factor 5 and angiotensin II receptor type I. Representative immunohistochemical images [CTL (wild-type), vehicle and cardiosphere-derived cell-treated Gαq mouse hearts stained for fibroblast-specific protein, KLF5, and AT-R1] and representative western blots and pooled data from Gαq mice treated at 8 weeks of age (*A* and *B*). Cardiosphere-derived cell treatment diminished co-localization of fibroblast-specific protein with KLF5 (*A*) and AT-R1 (*B*) and decreased AT-R1 and KLF5 protein levels (*A* and *B*). Arrows point to up-regulated KLF5 co-localized with fibroblast-specific protein in vehicle-treated Gαq mouse heart. Membranes were stripped and GAPDH probed as a control for loading in each lane. Data are means ± SEM; $n = 6–8$ in each group. $^{\dagger}P < 0.005$ vs. Gαq+CDC and control (CTL; wild-type). Scale bars: 10 μm.

contrast, 1 week after CDC treatment, MMP9 activity decreased relative to vehicle-treated or wild-type mice. This dynamic pattern may reflect dual effects of CDCs on MMP9 activity, with the early increase contributing to collagen degradation, and the later reduction promoting survival and differentiation of cardiac stem cells as previously shown.[23]

Cardiomyogenesis and cardiac lineage differentiation

Cardiomyocyte cycling and proliferation are infrequent in the normal adult mouse heart, as illustrated here by the low numbers of Ki67$^+$ and aurora B$^+$ cardiomyocytes (*Figure 7A* and *B* and CTL bars in pooled data). The vehicle-treated Gαq myocardium exhibits a several-fold increase in the numbers of Ki67$^+$ and aurora B$^+$ cardiomyocytes (*Figure 7A* and B and middle bars in pooled data), presumably as a compensatory mechanism in response to the enhanced apoptosis (*Figure 4B* and *D*). Cardiosphere-derived cells increase endogenous cardiomyogenesis in ischaemic models.[1–6] Likewise, CDC treatment promoted cardiomyogenesis in the Gαq model of DCM, as evidenced by a marked increase in Ki67$^+$ and aurora B$^+$ cardiomyocytes [*Figure 7A* and *B* and (Gq+CDC) bars in pooled data]. Previous work from our laboratory[1,24] implicates paracrine signalling by CDCs in the mechanism of these effects, consistent with the findings here of evanescent transplanted cell survival (Supplementary material online, *Figure S3*). In addition to enhanced cardimyogenesis, CDC treatment markedly increased cardiac lineage differentiation in the c-kit$^+$ cells [*Figure 7C* and (Gq+CDC) bar in the pooled data]. The number of c-kit$^+$ cells positive for Nkx2.5, an early cardiac transcription factor, was markedly higher in the CDC-treated hearts than in the other groups.

Survival analysis after adriamycin stress

Mortality is known to be enhanced in Gαq mice: the median survival is ~1.5 years, whereas 78% of control mice survive >2 years.[8] We accelerated heart failure progression even further by treating the mice with adriamycin. After ~4 months, all vehicle-treated Gαq mice had died, whereas >60% of CDC-treated Gαq (or control) mice remained alive (*Figure 8A*). Survival was significantly attenuated in vehicle-treated Gαq mice relative to either CDC-treated Gαq mice or wild-type controls; the latter two groups, however, were statistically indistinguishable.

Nrf2-Keap1 antioxidant defence pathway

In physiological settings, cells respond to oxidative stress by activating the Nrf2-Keap1 antioxidant defence pathway.[25] In vehicle-treated Gαq mice, despite severe oxidative/nitrosative stress (*Figure 2*), Nrf2 activity was impaired as evidenced by decreased nuclear content of Nrf2, increased cytoplasmic Keap1 protein density and, diminished expression of Nrf2 target gene products, catalase, and copper-zinc superoxide dismutase (Cu-Zn SOD). In contrast, CDC treatment restored the protein abundance of nuclear Nrf2,

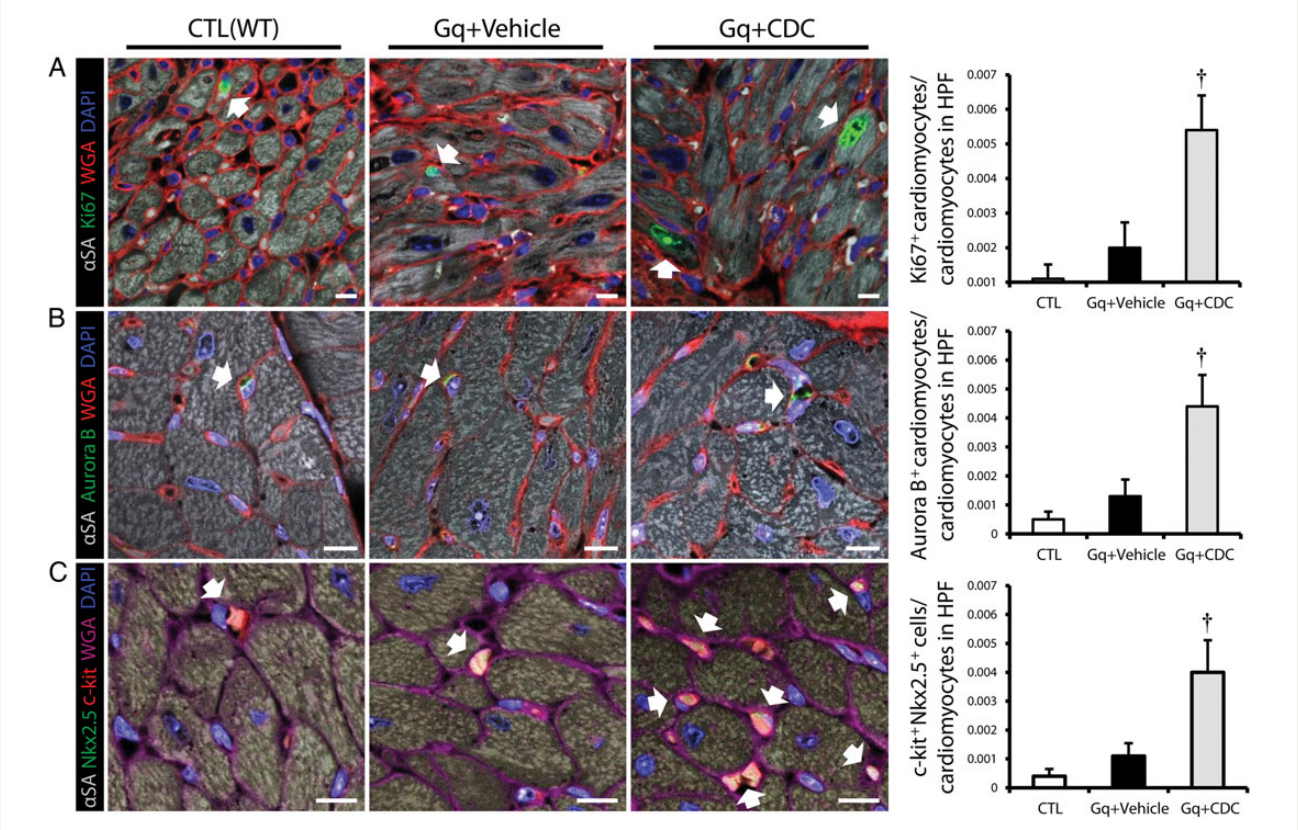

Figure 7 Cardiosphere-derived cell treatment increased cardiomyocyte cycling and proliferation and augmented number of c-kit positive cells differentiating into cardiac lineage (c-kit$^+$Nkx2.5$^+$). Representative immunohistochemical images and pooled data [*A*–*C*; CTL (wild-type), vehicle and cardiosphere-derived cell-treated Gαq mouse hearts stained for Ki67 (*A*), aurora B (*B*), c-kit and Nkx2.5 (*C*)] from Gαq mice treated at 8 weeks of age. Arrows point to Ki67$^+$ (*A*) and aurora B$^+$ (*B*) cardiomyocytes and the cells positive for both c-kit and Nkx2.5 (*C*). Fractions of cycling (Ki67$^+$) and proliferating (Aurora B$^+$) cardiomyocytes are expressed as the number of Ki67$^+$ and aurora B$^+$ cardiomyocytes divided by the total number of cardiomyocytes per high-power field, respectively [pooled data (*A*) and (*B*)]. The portion of c-kit$^+$Nkx2.5$^+$ cells was calculated as the number of c-kit$^+$Nkx2.5$^+$ cells divided by the total number of cardiomyocytes per HPF [pooled data (*C*)]. Wheat germ agglutinin was applied for staining and delineation of cell membrane. Data are means ± SEM; $n = 6–8$ in each group. $^{\dagger}P < 0.01$ vs. Gαq+Vehicle and control (CTL; wild-type); scale bars: 10 μm.

cytoplasmic Keap1, catalase, and Cu-Zn SOD to control levels (*Figure 8B*).

Discussion

Figure 8C depicts schematically the various disease processes at play in the Gαq mouse, and how CDCs impact each of these. Intramyocardial administration of CDCs in a mouse model of DCM preserves cardiac function; improves survival; blunts oxidative stress, inflammation and remodelling; and enhances cardiomyogenesis. This is accompanied by lower levels of ROS- and NO-generating enzymes, mitigation of inflammatory and remodelling pathways, deactivation of pro-apoptotic and activation of pro-survival mediators, marked reduction in fibrosis and significant augmentation of cycling and proliferating cardiomyocytes in the CDC-treated Gαq mouse heart.

Changes in cell signalling

Administration of CDCs reversed the Gαq-induced up-regulation of ROS- and NO-generating enzymes, prevented activation of inflammatory pathways, markedly diminished infiltration of inflammatory cells and significantly enhanced accumulation of reparative anti-inflammatory macrophages into cardiac tissue in Gαq mice. Such effects are consistent with the immunomodulatory properties recently described for CDCs.[26]

In physiological settings, cells respond to oxidative stress by activating the Nrf2-Keap1 pathway. Upon oxidative modification of the inhibitory subunit Keap1 or phosphorylation of Nrf2, Nrf2 dissociates from Keap1, translocates to the nucleus and up-regulates genes encoding many antioxidant and phase II detoxifying enzymes.[25] Despite severe oxidative/nitrosative stress, Nrf2 activity was impaired in Gαq mouse heart, a deficit reversed by CDCs (*Figure 8B*). It should be noted that, in CDC-treated Gαq mouse heart, protein abundance of phosphorylated/activated Akt was not only higher than that found in vehicle-treated Gαq mice, but also, even higher than the p-Akt content in wild-type mouse heart (*Figure 4D*). Given that Akt is among the protein kinases which phosphorylate Nrf2,[27] CDC-mediated reversal of impaired Nrf2 may be mediated, at least partly, via augmentation of active Akt.

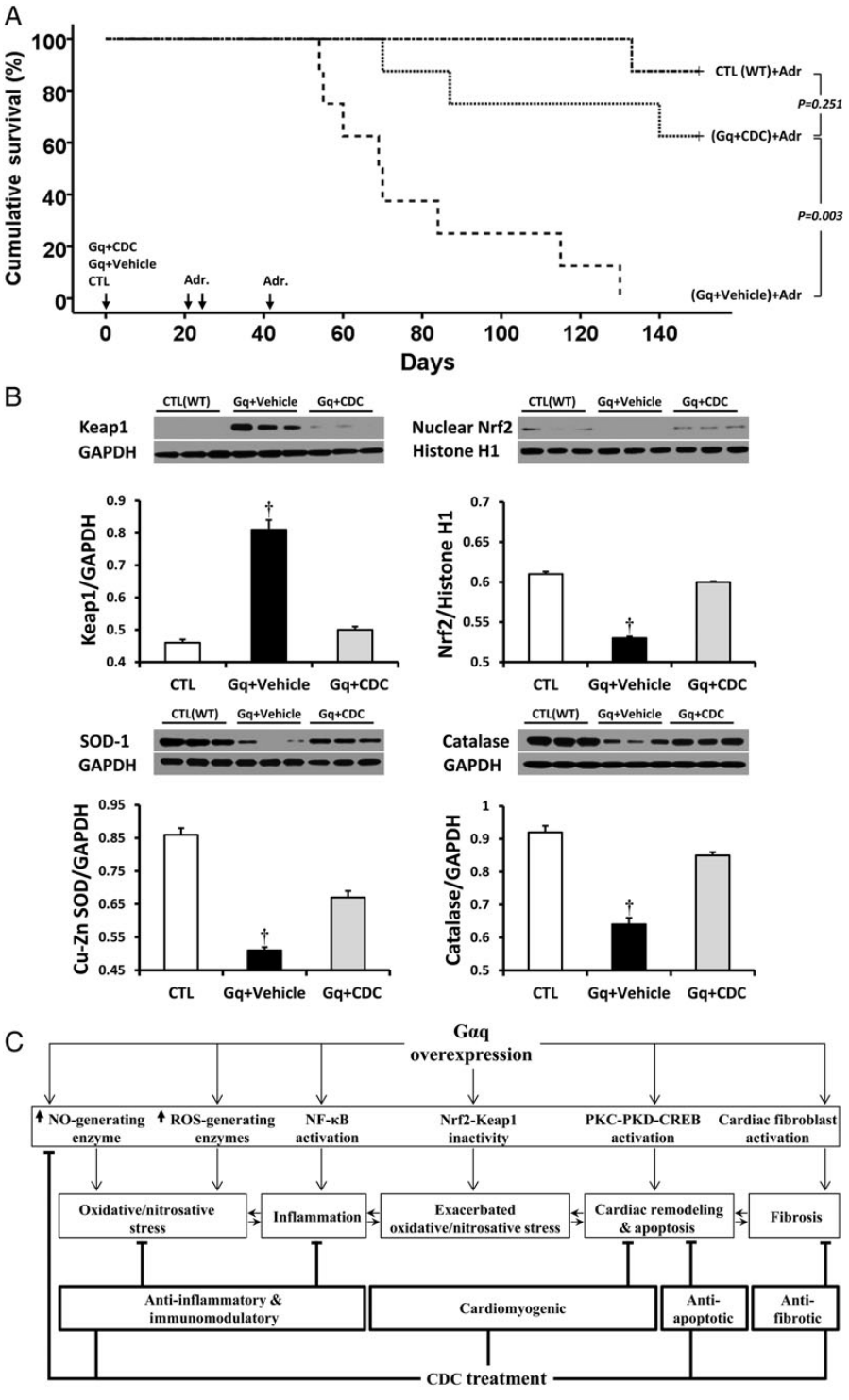

Figure 8 Survival analysis, changes in antioxidant defence pathway, and schematic summary of key findings. (*A*) Kaplan–Meier approach was applied for assessment of survival rate ($n = 8$ in each group). Survival was significantly attenuated in vehicle-treated Gαq mice relative to either cardiosphere-derived cell-treated Gαq mice or wild-type controls after adriamycin insult; the latter two groups, however, were statistically indistinguishable ($P < 0.001$, log-rank test). Cardiosphere-derived cell treatment restored activity of antioxidant pathway, Nrf2-Keap1. Representative western blots and pooled data from Gαq mice treated at 8 weeks of age demonstrate restored protein content of nuclear Nrf2, cytoplasmic Keap1 and the downstream gene products of Nrf2, catalase, and copper-zinc superoxide dismutase (Cu-Zn SOD), with cardiosphere-derived cell treatment. Membranes were stripped and GAPDH and histone H1 probed as a control for loading in each lane. Data are means ± SEM; $n = 6–8$ in each group. $^{\dagger}P < 0.005$ vs. Gαq+CDC and control (CTL; wild-type). (*C*) Various deleterious pathways are up-regulated in the Gαq model of dilated cardiomyopathy. Treatment with cardiosphere-derived cell impacted favourably on key disease processes including oxidative stress, inflammatory and remodelling pathways, apoptosis, and fibrosis.

Stimulation of the Gαq signalling pathway also exerts downstream effects via the PKC–PKD–CREB axis.[17] Activation of PKC and subsequent phosphorylation and nuclear translocation of PKD recruits CREB to target genes including those involved in adverse remodelling.[28–30] Of note, the roles of PKC–PKD–CREB signalling in pathological cardiac remodelling extend to inactivation of the pro-survival kinase, Akt kinase, and activation of the pro-apoptotic mediator, JNK.[18] In CDC-treated Gαq mice, CDC restored activity of PKC and its downstream effectors, PKD and CREB, prevented inactivation of Akt and activation of JNK, and blunted apoptosis.

Anti-fibrotic, anti-apoptotic, and cardioproliferative effects of cardiosphere-derived cells

Cardiac fibroblasts interact with adjacent cardiomyocytes to induce myocardial hypertrophy as an adaptive response to pressure overload. Among the mediators contributing to this interplay, KLF5 transactivates insulin-like growth factor 1 (IGF-1) in cardiac fibroblasts, and IGF1 subsequently induces hypertrophy in cardiomyocytes.[21] The expression of KLF5 in cardiac fibroblasts is regulated via angiotensin II signalling,[20] leading to overproduction of collagen and exacerbated fibrosis. In vehicle-treated Gαq mice, abundant collagen was found in cardiac tissue co-localized with cardiac fibroblasts and AT-R1 and KLF5, signifying active cardiac fibroblast induced-adaptive/maladaptive responses. Cardiosphere-derived cell treatment profoundly reduced collagen deposition and collagen abundance in co-localization with fibroblasts and decreased the expression of AT-R1 and KLF5. In addition to restoring KLF5 and AT-R1 protein levels, CDC treatment markedly increased cardiac MMP9 activity 1 day after treatment, highlighting the multivariate effects of CDCs on fibrosis.

We find that CDCs favourably influence diverse pathophysiological processes including fibrosis, apoptosis, cardiomyocyte proliferation, and c-kit$^+$ cell differentiation into cardiac lineage, although there is now strong evidence against the importance of c-kit$^+$ cells as cardiomyocyte progenitors.[31] Such a diversity of effects has been documented previously with cardiospheres and their progeny in models of acute myocardial infarction[32,33] and chronic ischaemic cardiomyopathy.[3,34] While the present study was designed to answer 'Do they work?' not 'Precisely how do they work?', it is nevertheless natural to wonder how one therapeutic entity can recruit so many diverse pathways (*Figure 8C*). Increasing evidence points to indirect mechanisms as dominant,[1,24,35] consistent with our observation that transplanted CDCs do not survive long term (Supplementary material online, *Figure S3*) and that CDC-conditioned media exerts beneficial effects on oxidant-stressed cardiomyocytes (Supplementary material online, *Figure S5*). Such mechanisms include not only a panoply of soluble protein factors,[36] but also microRNAs that are secreted in exosomes by CDCs.[37] Thus, the diversity of effects may reflect not only soluble protein factors (Supplementary material online, *Figure S7*) targeting different pathways, but also the ability of microRNAs to serve as master regulators of gene expression.[38] Further work will be required to pinpoint the mediators of the salutary effects of CDCs on murine DCM.

Temporal considerations of cardiosphere-derived cell benefit

The beneficial effects of CDCs on cardiac global function persisted for at least 3 months. This model, however, is one of constitutive expression of Gαq; there is good reason to expect that the benefits of CDCs may be transient, as newly generated myocytes themselves succumb to oxidative stress. Consistent with this prediction, cardiac 3-nitrotyrosine levels in CDC-treated mice were higher 3 months after treatment than at 3 weeks, although they still remained lower than in vehicle-treated mice (Supplementary material online, *Figure S8*). This finding hints that the beneficial effects of CDCs may wear off over time; if so, periodic re-administration of CDCs may be necessary to maintain longer-term functional improvement. Nevertheless, physiological deterioration is halted for at least 3 months, and survival, as one of the main treatment goals in heart failure,[39] is markedly enhanced when CDC-treated animals are challenged by a cardiotoxic insult. Why do the beneficial effects of CDCs last as long as they do? Among the various mechanisms, CDCs promote cardiomyocyte proliferation. The newly generated myocytes benefit from the antioxidant, anti-fibrotic, and anti-inflammatory milieu established, at least temporarily, by the CDCs; presumably it takes some time for these new myocytes to succumb to the progressive stress of Gαq overexpression, just as several weeks of postnatal life are required for cardiac dysfunction to become manifest in the first place.

Limitations

The present study has several important limitations. The mouse model of DCM, although well-studied and reproducible, may not be predictive for the human condition. The duration of observation post-treatment was limited to 3 months in the functional and tissue studies and to 4 months in the survival analysis; longer-term studies will be required to accurately define the time course of persistence of benefit, as well as the possible utility of repeat dosing. Here, we have used single dosing with wild-type mouse CDCs derived from the founder strain; we do not anticipate immunologic reactions in syngeneic therapy with highly inbred mice,[1,35] but we recognize that such reactions will need to be carefully assessed before repeat dosing of allogeneic products is contemplated in humans. Finally, the study was designed to determine whether CDCs are beneficial in the Gαq mouse model of DCM, and, if so, whether the core pathophysiological processes at play are influenced by CDCs. While we have addressed these questions, we have not attempted here to pinpoint the root mechanisms of benefit, other than to establish that the mechanisms must be indirect.

In summary, CDC treatment prevents functional deterioration, attenuates oxidative/nitrosative stress, promotes cardiomyocyte proliferation and reduces mortality in a transgenic mouse model of DCM.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: E.M. is founder and equity holder in Capricor, Inc. K.M. receives consulting income from Capricor. The other authors report no conflicts.

References

1. Malliaras K, Li TS, Luthringer D, Terrovitis J, Cheng K, Chakravarty T, Galang G, Zhang Y, Schoenhoff F, Van Eyk J, Marbán L, Marbán E. Safety and efficacy of allogeneic cell therapy in infarcted rats transplanted with mismatched cardiosphere-derived cells. *Circulation* 2012;**125**:100–112.
2. Li TS, Cheng K, Malliaras K, Smith RR, Zhang Y, Sun B, Matsushita N, Blusztajn A, Terrovitis J, Kusuoka H, Marbán L, Marbán E. Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells. *J Am Coll Cardiol* 2012;**59**:942–953.
3. Lee ST, White AJ, Matsushita S, Malliaras K, Steenbergen C, Zhang Y, Li TS, Terrovitis J, Yee K, Simsir S, Makkar R, Marbán E. Intramyocardial injection of autologous cardiospheres or cardiosphere-derived cells preserves function and minimizes adverse ventricular remodeling in pigs with heart failure post-myocardial infarction. *J Am Coll Cardiol* 2011;**57**:455–465.
4. Cheng K, Malliaras K, Li TS, Sun B, Houde C, Galang G, Smith J, Matsushita N, Marbán E. Magnetic enhancement of cell retention, engraftment, and functional benefit after intracoronary delivery of cardiac-derived stem cells in a rat model of ischemia/reperfusion. *Cell Transplant* 2012;**21**:1121–1135.
5. Cheng K, Li TS, Malliaras K, Davis DR, Zhang Y, Marbán E. Magnetic targeting enhances engraftment and functional benefit of iron-labeled cardiosphere-derived cells in myocardial infarction. *Circ Res* 2010;**106**:1570–1581.
6. Makkar RR, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marbán L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marbán E. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. *Lancet* 2012;**379**:895–904.
7. D'Angelo DD, Sakata Y, Lorenz JN, Boivin GP, Walsh RA, Liggett SB, Dorn GW II. Transgenic Galphaq overexpression induces cardiac contractile failure in mice. *Proc Natl Acad Sci USA* 1997;**94**:8121–8126.
8. Roth DM, Bayat H, Drumm JD, Gao MH, Swaney JS, Ander A, Hammond HK. Adenylyl cyclase increases survival in cardiomyopathy. *Circulation* 2002;**105**:1989–1994.
9. Adams JW, Sakata Y, Davis MG, Sah VP, Wang Y, Liggett SB, Chien KR, Brown JH, Dorn GW II. Enhanced galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. *Proc Natl Acad Sci USA* 1998;**95**:10140–10145.
10. Qin F, Lennon-Edwards S, Lancel S, Biolo A, Siwik DA, Pimentel DR, Dorn GW, Kang YJ, Colucci WS. Cardiac-specific overexpression of catalase identifies hydrogen peroxide-dependent and -independent phases of myocardial remodeling and prevents the progression to overt heart failure in G(alpha)q-overexpressing transgenic mice. *Circ Heart Fail* 2010;**3**:306–313.
11. Satoh M, Matter CM, Ogita H, Takeshita K, Wang CY, Dorn GW 2nd, Liao JK. Inhibition of apoptosis-regulated signaling kinase-1 and prevention of congestive heart failure by estrogen. *Circulation* 2007;**115**:3197–3204.
12. Trachtenberg BH, Hare JM. Biomarkers of oxidative stress in heart failure. *Heart Fail Clin* 2009;**5**:561–577.
13. Malliaras K, Marbán E. Cardiac cell therapy: Where we've been, where we are, and where we should be headed. *Br Med Bull* 2011;**98**:161–185.
14. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marbán E. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation* 2007;**115**:896–908.
15. Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, Crystal RG, Darnell RB, Ferrante RJ, Fillit H, Finkelstein R, Fisher M, Gendelman HE, Golub RM, Goudreau JL, Gross RA, Gubitz AK, Hesterlee SE, Howells DW, Huguenard J, Kelner K, Koroshetz W, Krainc D, Lazic SE, Levine MS, Macleod MR, McCall JM, Moxley RT III, Narasimhan K, Noble LJ, Perrin S, Porter JD, Steward O, Unger E, Utz U, Silberberg SD. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature* 2012;**490**:187–191.
16. Cheng K, Blusztajn A, Shen D, Li TS, Sun B, Galang G, Zarembinski TI, Prestwich GD, Marbán E, Smith RR, Marbán L. Functional performance of human cardiosphere-derived cells delivered in an *in situ* polymerizable hyaluronan-gelatin hydrogel. *Biomaterials* 2012;**33**:5317–5324.
17. Ozgen N, Obreztchikova M, Guo J, Elouardighi H, Dorn GW 2nd, Wilson BA, Steinberg SF. Protein kinase D links Gq-coupled receptors to cAMP response element-binding protein (CREB)-Ser133 phosphorylation in the heart. *J Biol Chem* 2008;**283**:17009–17019.
18. Ben-Ami I, Yao Z, Naor Z, Seger R. Gq protein-induced apoptosis is mediated by AKT kinase inhibition that leads to protein kinase C-induced c-Jun N-terminal kinase activation. *J Biol Chem* 2011;**286**:31022–31031.
19. Goldsmith EC, Bradshaw AD, Spinale FG. Cellular mechanisms of tissue fibrosis. 2. Contributory pathways leading to myocardial fibrosis: Moving beyond collagen expression. *Am J Physiol Cell Physiol* 2013;**304**:C393–C402.
20. Shindo T, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, Kawai-Kowase K, Moriyama N, Imai Y, Kawakami H, Nishimatsu H, Ishikawa T, Suzuki T, Morita H, Maemura K, Sata M, Hirata Y, Komukai M, Kagechika H, Kadowaki T, Kurabayashi M, Nagai R. Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nat Med* 2002;**8**:856–863.
21. Takeda N, Manabe I, Uchino Y, Eguchi K, Matsumoto S, Nishimura S, Shindo T, Sano M, Otsu K, Snider P, Conway SJ, Nagai R. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. *J Clin Invest* 2010;**120**:254–265.
22. Vellaichamy E, Khurana ML, Fink J, Pandey KN. Involvement of the NF-kappa B/matrix metalloproteinase pathway in cardiac fibrosis of mice lacking guanylyl cyclase/natriuretic peptide receptor A. *J Biol Chem* 2005;**280**:19230–19242.
23. Mishra PK, Chavali V, Metreveli N, Tyagi SC. Ablation of MMP9 induces survival and differentiation of cardiac stem cells into cardiomyocytes in the heart of diabetics: a role of extracellular matrix. *Can J Physiol Pharmacol* 2012;**90**:353–360.
24. Chimenti I, Smith RR, Li TS, Gerstenblith G, Messina E, Giacomello A, Marbán E. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. *Circ Res* 2010;**106**:971–980.
25. Jeong WS, Jun M, Kong AN. Nrf2: A potential molecular target for cancer chemoprevention by natural compounds. *Antioxid Redox Signal* 2006;**8**:99–106.
26. Lauden L, Boukouaci W, Borlado LR, Lopez IP, Sepulveda P, Tamouza R, Charron D, Al-Daccak R. Allogenicity of human cardiac stem/progenitor cells orchestrated by programmed death ligand 1. *Circ Res* 2013;**112**:451–464.
27. Wang L, Chen Y, Sternberg P, Cai J. Essential roles of the PI3 kinase/Akt pathway in regulating Nrf2-dependent antioxidant functions in the RPE. *Invest Ophthalmol Vis Sci* 2008;**49**:1671–1678.
28. Fielitz J, Kim MS, Shelton JM, Qi X, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. Requirement of protein kinase D1 for pathological cardiac remodeling. *Proc Natl Acad Sci USA* 2008;**105**:3059–3063.
29. Ichiki T. Role of cAMP response element binding protein in cardiovascular remodeling: good, bad, or both? *Arterioscler Thromb Vasc Biol* 2006;**26**:449–455.
30. Waldron RT, Innamorati G, Torres-Marquez ME, Sinnett-Smith J, Rozengurt E. Differential PKC-dependent and -independent PKD activation by G protein α subunits of the Gq family: selective stimulation of PKD Ser^{748} autophosphorylation by Gαq. *Cell Signal* 2012;**24**:914–921.
31. Van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SJ, Middleton RC, Marbán E, Molkentin JD. c-kit^{+} cells minimally contribute cardiomyocytes to the heart. doi:10.1038/nature13309
32. Cheng K, Shen D, Smith J, Galang G, Sun B, Zhang J, Marbán E. Transplantation of platelet gel spiked with cardiosphere-derived cells boosts structural and functional benefits relative to gel transplantation alone in rats with myocardial infarction. *Biomaterials* 2012;**33**:2872–2879.
33. Malliaras K, Zhang Y, Seinfeld J, Galang G, Tseliou E, Cheng K, Sun B, Aminzadeh M, Marbán E. Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart. *EMBO Mol Med* 2013;**5**:191–209.
34. Johnston PV, Sasano T, Mills K, Evers R, Lee ST, Smith RR, Lardo AC, Lai S, Steenbergen C, Gerstenblith G, Lange R, Marbán E. Engraftment, differentiation, and functional benefits of autologous cardiosphere-derived cells in porcine ischemic cardiomyopathy. *Circulation* 2009;**120**:1075–1083, 1077 p following 1083.
35. Tseliou E, Pollan S, Malliaras K, Terrovitis J, Sun B, Galang G, Marbán L, Luthringer D, Marbán E. Allogeneic cardiospheres safely boost cardiac function and attenuate adverse remodeling after myocardial infarction in immunologically mismatched rat strains. *J Am Coll Cardiol* 2013;**61**:1108–1119.
36. Stastna M, Van Eyk JE. Secreted proteins as a fundamental source for biomarker discovery. *Proteomics* 2012;**12**:722–735.
37. Ibrahim AG, Cheng K, Marbán E. Exosomes as Critical Agents of Cardiac Regeneration Triggered by Cell Therapy. *Stem Cell Reports* 2014, http://dx.doi.org/10.1016/j.stemcr.2014.04.006.

38. Sun W, Julie Li YS, Huang HD, Shyy JY, Chien S. microRNA: a master regulator of cellular processes for bioengineering systems. *Annu Rev Biomed Eng* 2010;**12**: 1–27.
39. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Böhm M, Dickstein K, Falk V, Filippatos G, Fonseca C, Gomez-Sanchez MA, Jaarsma T, Køber L, Lip GY, Maggioni AP, Parkhomenko A, Pieske BM, Popescu BA, Rønnevik PK, Rutten FH, Schwitter J, Seferovic P, Stepinska J, Trindade PT, Voors AA, Zannad F, Zeiher A. ESC Committee for Practice Guidelines. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur Heart J* 2012;**33**:1787–1847.

CARDIOVASCULAR FLASHLIGHT

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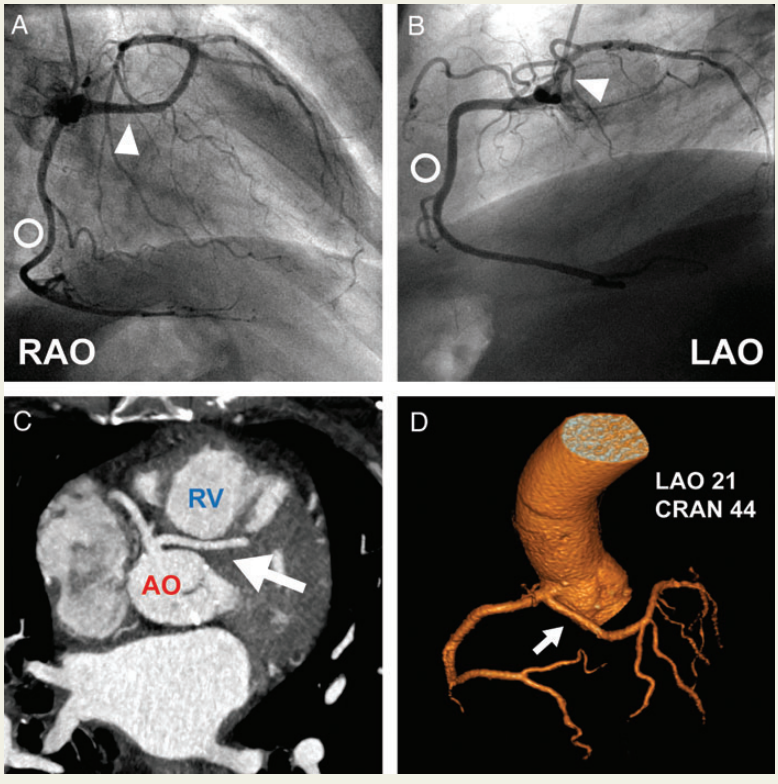
Single coronary artery anomaly with interarterial left main: caught inbetween

L. Christian Napp[1]*, Imke Schatka[2], Tibor Kempf[1], Udo Bavendiek[1], Frank M. Bengel[2], and Johann Bauersachs[1]

[1]Department of Cardiology and Angiology OE 6880, Hannover Medical School, Carl-Neuberg-Str. 1, Hannover 30625, Germany and [2]Department of Nuclear Medicine, Hannover Medical School, Carl-Neuberg-Str. 1, Hannover 30625, Hannover, Germany

*Corresponding author. Tel: +49 176 1532 5735, Fax: +49 511 532 5412, Email: napp.christian@mh-hannover.de



An 82-year-old patient with suspected pulmonary hypertension was referred to our department for cardiac catheterization. Right heart catheterization confirmed precapillary pulmonary hypertension. Coronary angiography revealed that the left main (LMCA, arrowhead) originated from the right sinus of valsalva in a common ostium together with the RCA (circle) (*Panels A* and *B*, Supplementary material online, *Videos S1* and *S2*). Computed tomography confirmed that the LMCA (arrow) passed between the aorta (AO) and the right ventricular (RV) outflow tract and gave rise to the LAD before running towards the lateral wall as LCX (*Panels C* and *D*). Computed tomography confirmed the absence of an additional coronary artery, thus the patient had a congenital single coronary artery anomaly with an interarterial left main.

Single coronary artery anomaly has an estimated prevalence of $<0.1\%$ in the general population. Various subtypes are classified depending on the origin and route of coronary arteries. Our patient had subtype RII-B according to the nomenclature published by Lipton et al. in 1979. In cases with an interarterial course, the coronary artery is prone to be compressed between the great arteries. Such patients are at increased risk for myocardial infarction and sudden death even in the absence of atherosclerosis, e.g. during exercise or other situations great artery enlargement such as pulmonary hypertension. Surgical revascularization may be indicated for prevention of sudden death according to current guidelines. In our case, due to the age of the patient we recommended a conservative strategy and advised to avoid intensive physical exercise. Once coronary artery anomalies are found, computed tomography should be performed to unequivocally determine anatomical relations.

Supplementary Material is available at *European Heart Journal* online.