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Adenoviral Mediated Delivery of OSKM Factors Induces Partial Reprogramming of Mouse Cardiac Cells In Vivo

Thomas Kisby, Irene de Lázaro, Sudeshna Fisch, Elizabeth J. Cartwright, Giulio Cossu, and Kostas Kostarelos**

The induction of in vivo reprogramming toward pluripotency has been demonstrated in several tissues utilizing either transgenic inducible mice or gene delivery approaches. However, the effects of exogenous reprogramming factor expression in the mammalian heart have not been previously reported. The present study aims to investigate the response of cardiac cells to ectopic *Oct3/4*, *Sox2*, *Klf4*, and *cMyc* (OSKM) expression in vivo using a non-integrating adenoviral vector. Direct intramyocardial injection of this vector achieves effective and transient OSKM overexpression in the healthy heart and after myocardial infarction. The expression of these factors induces transient upregulation of a number of endogenous pluripotency (*endo-Oct3/4*, *Gdf3*) and reprogramming related (*Cdh1*, *Fut4*) genes, confirming the induction of cell reprogramming. Despite the initiation of reprogramming, markers of fully de-differentiated cells including *Nanog* remain silenced, consistent with a partially reprogrammed state. Furthermore, no indications of tumorigenesis or teratoma formation are observed. Overall, these data suggest that adenoviral mediated OSKM delivery can be utilized to induce partial in vivo reprogramming. However, the absence of any clear regenerative effects after myocardial infarction indicates that further optimization of vector mediated reprogramming strategies is essential to overcome barriers to therapeutic efficacy.

1. Introduction

The forced expression of a defined combination of transcription factors; *Oct3/4*, *Sox2*, *Klf4*, and *cMyc* (OSKM) has been uncovered as a strategy to induce dedifferentiation of somatic cells toward pluripotency both in vitro[1] and in the in vivo microenvironment.[2–4] In both cases, sustained overexpression of such factors can lead to the generation of stable, induced pluripotent stem cells (iPSCs) which in vivo give rise to teratomas.[1,2] However, temporally limited OSKM expression can instead generate proliferative, partially reprogrammed intermediates with incomplete loss of epigenetic identity and a restricted differentiation capacity.[5–7] Transient or cyclic expression of OSKM in vivo has been demonstrated to induce partial and/or transient reprogramming of cells within a number of tissues including liver,[3,4] skeletal muscle,[8] brain,[9,10] skin,[11] optic nerve,[12] kidney, and pancreas.[13,14] Provided OSKM expression is not sustained, these partially reprogrammed or pluripotent-like cells appear to maintain the

Dr. T. Kisby, Dr. I. de Lázaro, Prof. K. Kostarelos
Nanomedicine Lab
Faculty of Biology
Medicine and Health
AV Hill Building
The University of Manchester
Manchester M13 9PT, UK
E-mail: idelazaro@g.harvard.edu; kostas.kostarelos@manchester.ac.uk

Dr. I. de Lázaro
Harvard John A. Paulson School of Engineering and Applied Sciences
58 Oxford Street
Cambridge, MA 02138, USA

Dr. I. de Lázaro
Wyss Institute for Biologically Inspired Engineering at Harvard University
Center for Life Science
3 Blackfan Circle, Boston, MA 02115, USA

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adtp.202000141

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Dr. S. Fisch
Cardiovascular Physiology Core
Brigham and Women's Hospital
Harvard Medical School
Boston, MA 02115, USA

Dr. E. J. Cartwright
Division of Cardiovascular Sciences
Faculty of Biology
Medicine and Health
Manchester Academic Health Science Centre
The University of Manchester
Manchester M13 9PL, UK

Prof. G. Cossu
Division of Cell Matrix Biology and Regenerative Medicine
Faculty of Biology
Medicine and Health
The University of Manchester
Manchester M13 9PT, UK

Prof. K. Kostarelos
Catalan Institute of Nanoscience and Nanotechnology (ICN2)
Campus UAB
Bellaterra, Barcelona 08193, Spain

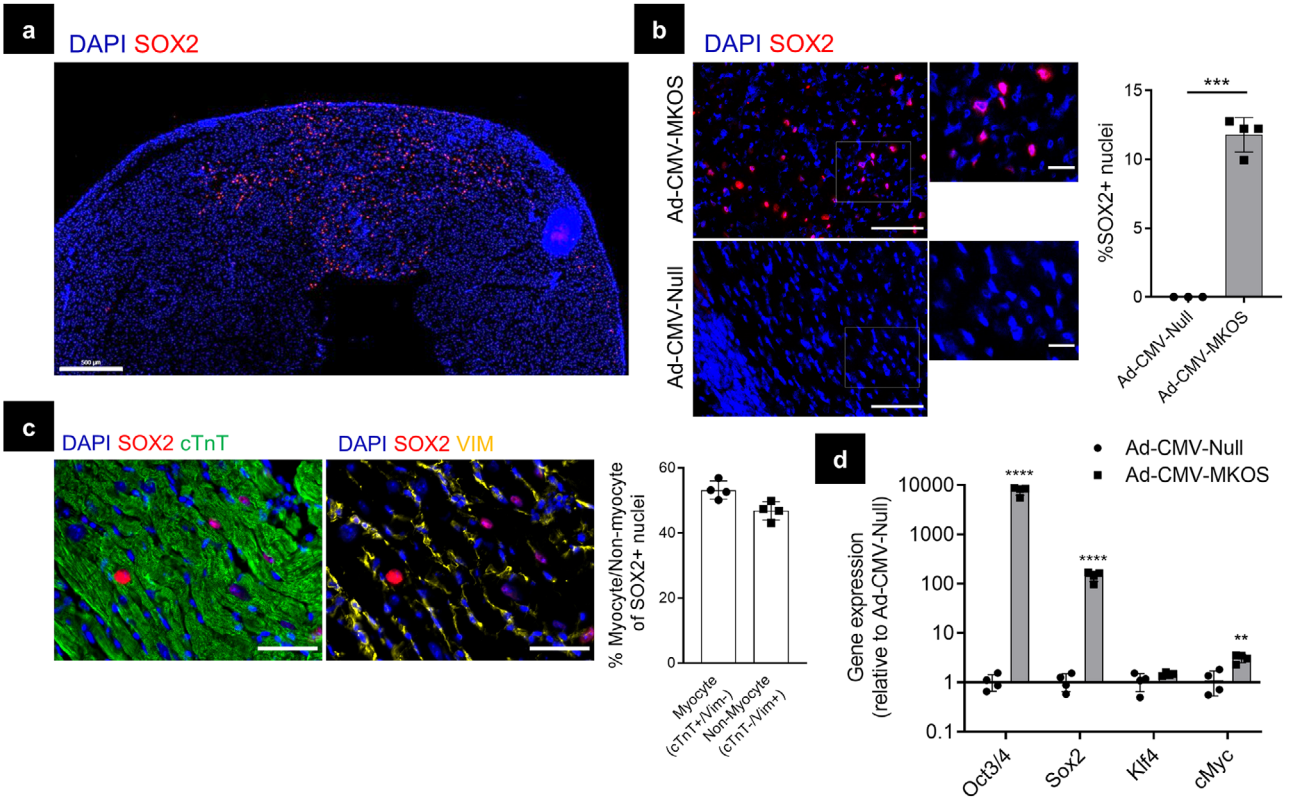

Figure 1. Effective overexpression of OSKM factors in cardiac cells in vivo. a) Localization of SOX2+ cells following single intramyocardial injection of Ad-CMV-MKOS (Scale bar = 500 μm). b) Quantification of total SOX2+ cells within the injection site of hearts 3 days after injection with Ad-CMV-MKOS and Ad-CMV-Null (Scale bars = 100 μm and 25 μm, n = 3/4, 4 fields per replicate). c) Quantification of the % of myocytes (cTnT+/Vim−) and non-myocytes (Vim+/cTnT−) out of total SOX2+ cells 3 days after injection of Ad-CMV-MKOS (Scale bar = 50 μm, n = 4, 3–4 fields per replicate). d) Relative expression of OSKM in the left ventricle on day 6 post injection with Ad-CMV-MKOS (n = 4). Data presented as mean ± S.D. b) Unpaired t-test with Welch's correction, d) Unpaired t-test, **, ***, and **** denote $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

capacity to spontaneously re-differentiate and contribute to enhanced regeneration in injury models.[8,9,13] Notably, the cardiac regenerative capacity of lower vertebrates has been attributed to the capacity of cardiomyocytes to undergo dedifferentiation, followed by proliferation and redifferentiation, which shares many similarities to the induced transient reprogramming described above.[15–17] However, despite the clinical need for novel cardiac regenerative therapies, reprogramming of cardiac cells toward pluripotency in situ has not yet been investigated.

The majority of previous investigations of both transient and sustained in vivo reprogramming toward pluripotency have relied on doxycycline inducible OSKM transgenic reprogrammable mice.[2,9,11,14,18,19] Although highly valuable for exploring the biological effects of systemic OSKM overexpression, they are limited both in terms of the clinical translatability of the findings and the challenges associated with targeting OSKM overexpression to a particular tissue or organ. Investigations that have utilized exogenous gene delivery for in vivo reprogramming have been limited by either the efficiency of the delivery vehicle[4,8] or the integrating nature of vectors leading to sustained reprogramming and teratoma formation.[20,21] It is therefore essential that alternative gene delivery vectors are explored for potential in vivo applications of transient and partial reprogramming. Despite the known efficiency and transiency of adenoviral mediated gene delivery, the use of this vector for in vivo reprogramming toward pluripotency has not been previously reported. Here, we investigated the effect of exogenous OSKM expression in the adult mammalian heart using a non-integrating adenoviral vector.

2. Results

2.1. Effective Ectopic OSKM Expression in the Myocardium Following Direct Injection of Adenovirus

To investigate the effects of OSKM overexpression in the heart we utilized an adenoviral vector containing all four factors in a single polycistronic expression cassette (Ad-CMV-MKOS). Effective delivery and expression of OSKM was confirmed 3 days following direct intramyocardial injection of 1×10^8 IFU by the presence of SOX2 positive cells across the anterior wall of the left ventricle (**Figure** 1a). The percentage of nuclei positive for SOX2 within this transduced region was calculated to be 11.8 ± 1.2% which included both myocytes and non-myocytes (Figure 1b,c). As expected, no SOX2 positive cells were identified in any hearts injected with a control (Ad-CMV-Null) vector (Figure 1b). Overexpression of the reprogramming factors relative to control was further confirmed by real-time RT-qPCR on

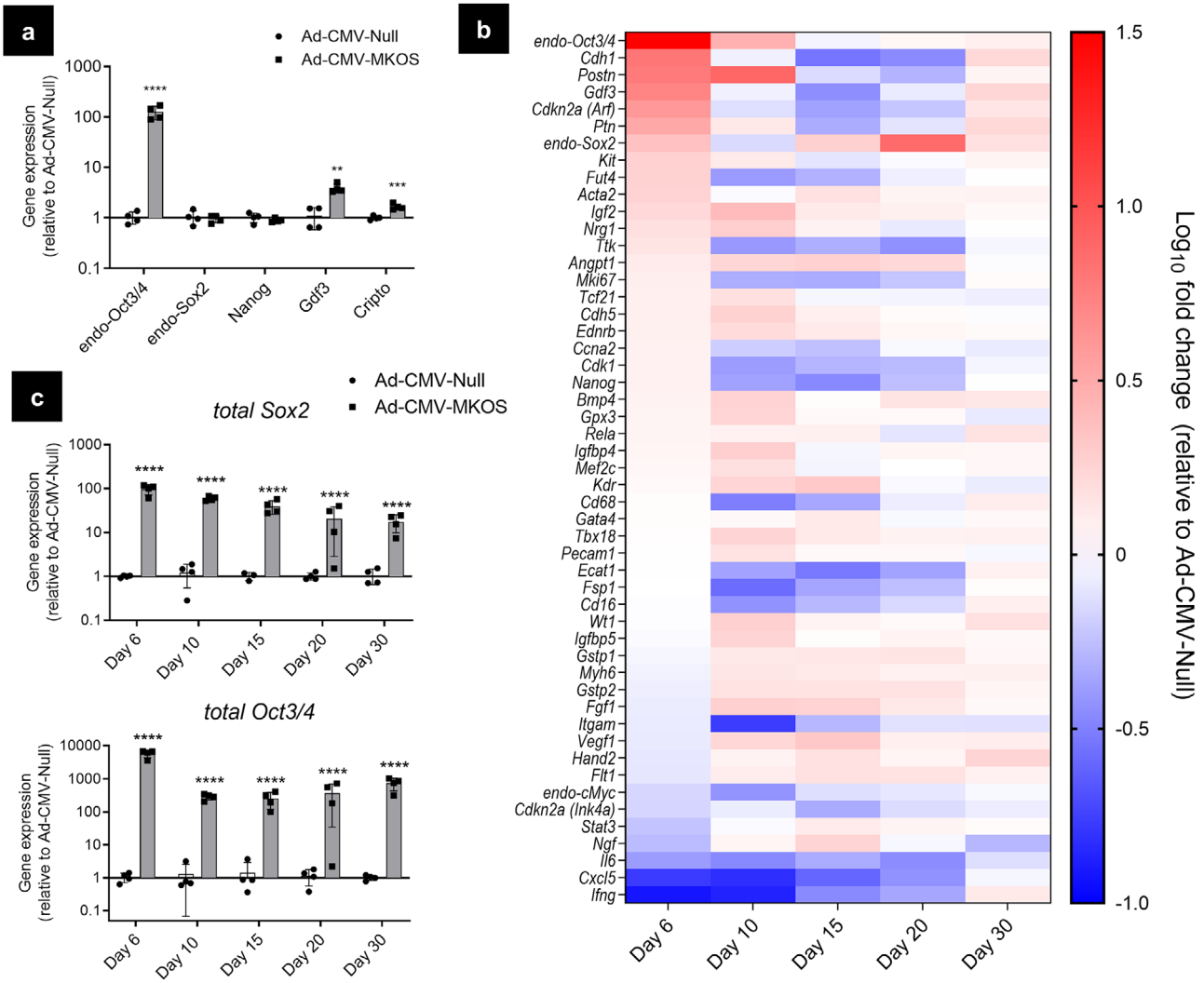

Figure 2. OSKM induces transient upregulation of endogenous pluripotency genes. a) Relative expression of endogenous pluripotency genes in the left ventricle day 6 post injection with Ad-CMV-MKOS ($n = 4$). b) Heatmap of significant gene expression changes between Ad-CMV-MKOS ($n = 3$–4) and Ad-CMV-Null ($n = 3$–4) injected hearts on days 6, 10, 15, 20 and 30 following intramyocardial injection. c) Relative expression of *total Sox2* and *Oct3/4* on days 6–30 post injection with Ad-CMV-MKOS ($n = 3$–4). Data presented as mean ± S.D (a,c). Data presented as $\log_{10}$ fold change relative to Ad-CMV-Null (b). Unpaired *t*-test (a), Two-way ANOVA with Bonferroni's correction (b,c), **, ***, and **** denote $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

days 3 and 6 post injection (Figure 1d and Figure S1a, Supporting Information). This confirmed the suitability of this vector mediated strategy to induce OSKM overexpression locally in the myocardium.

2.2. Overexpression of OSKM Induces Upregulation of Endogenous Pluripotency Related Genes

We next investigated the transcriptional response of cardiac cells to adenoviral mediated OSKM expression. On day 6 post injection significant upregulation of several endogenous pluripotency related genes was identified including *endogenous Oct3/4, Gdf3*, and *Cripto (Tdgf1)* (**Figure 2**a) which corresponded with the peak of exogenous *OSKM* gene expression (Figure S1a, Supporting Information). These genes are expressed almost exclusively in embryonic cells and are epigenetically silenced in differentiated tissues,[22,23] thus indicating the induction of a reprogramming response within the myocardium. However, progression of cells toward pluripotency appeared incomplete as both *endogenous Sox2* and *Nanog* remained unchanged at this timepoint. While increasing the dose of vector had modest effects on *OSKM* overexpression, this did not lead to further increases in the expression of endogenous *Oct3/4, Gdf3* and *Cripto* and did not lead to upregulation of *Nanog* (Figure S1b,c, Supporting Information).

We further investigated the downstream effects of exogenous OSKM expression over time by analyzing a larger panel of genes using high throughput dynamic array RT-qPCR on days 6, 10, 15, 20, and 30 post injection. The most suitable reference genes for

RT-qPCR normalization were identified by assessing the stability of 10 commonly reported reference genes within these samples (Table S1, Supporting Information). The genes of interest for this investigation were selected from the literature based on their relevance to cell reprogramming, pluripotency, cardiac development, repair, and inflammation. Of the 77 genes included, 51 showed a significant difference relative to Ad-CMV-Null injected hearts in at least one of the timepoints investigated (Figure 2b, Table S2, Supporting Information). Consistent with the induction of cell reprogramming, the genes that were most upregulated on day 6 after injection were those associated with pluripotency (*endogenous Oct3/4*, *Gdf3*) and the early (initiation) phases of reprogramming (*Cdh1, Kit, Fut4*) (Figure 2b, Table S2, Supporting Information).[24] However, these genes were not significantly increased above control levels at any of the other timepoints investigated, demonstrating the transiency of adenoviral mediated reprogramming. Notably, while most endogenous genes returned to control levels, significantly elevated levels of *total Sox2* and *total Oct3/4* mRNA (largely exogenous) could be identified at all timepoints, suggesting the transient nature of this response is not due to rapid elimination of adenoviral transduced cells (Figure 2c). However, while mRNA was readily detected, SOX2 protein appeared more rapidly silenced which is likely responsible for the transiency of these downstream responses (Figure S1d, Supporting Information). Although some pluripotency related genes appeared upregulated at early time points after injection, and endogenous Sox2 was upregulated 20 days after injection, this did not appear to lead to the establishment of a complete pluripotency gene network since *Nanog* and *Ecat1* were unchanged even during the initial response (Figure 2b, Table S2, Supporting Information). Furthermore, no significant downregulation in cardiomyocyte differentiation related genes (*Myh6*, *Myh7*) or genes pointing to other cardiac cell types (*Fsp1*, *Vim*, *Pecam1*) were identified at any timepoint suggesting dedifferentiation was limited such that cells did not lose their initial identity, consistent with partial reprogramming.[5,13,25] However, it is possible that changes in cardiac gene expression in a small number of cells would not be detected using the bulk tissue RNA analysis utilized here, due to dilution effects. Interestingly, pluripotency genes (*Nanog, Ecat1*), along with several cell cycle related genes (*Mki67*, *Cdk1*, *Ttk*, *endogenous cMyc*, *Ccna2*) were significantly downregulated relative to control at later timepoints (days 10 to 15) post injection indicating a potential endogenous negative regulation in response to OSKM overexpression (Figure 2b). Additionally, as reported by others, the senescence locus *Cdkn2a (Arf)* was upregulated on day 6 coinciding with the induction of *endogenous Oct3/4*, which may contribute to this downstream effect on cell cycle genes.[19] In further support of the transiency and incomplete nature of this reprogramming response, we did not identify teratomas in any hearts at the gross or histological level (Figure S2a,b, Supporting Information) or in any other organ assessed at the gross tissue level during necropsy, up to 4 months post Ad-CMV-MKOS injection. This is supported by animal weight which was not different to control vector injected mice at any timepoint investigated (Figure S2c, Supporting Information). Taken together, these data suggest that direct intramyocardial injection of Ad-CMV-MKOS induces partial and transient reprogramming in the adult mammalian heart.

2.3. Effects of OSKM Overexpression in the Injured Myocardium

The tissue microenvironment has been demonstrated to strongly influence the efficiency and progression of OSKM mediated reprogramming to pluripotency in vivo.[18,19,26] In particular, the presence of injury and inflammatory signals have been shown to have profound supportive actions on the progression of in vivo reprogramming in transgenic reprogrammable mice under systemic OSKM induction protocols.[18,19,26] Therefore, to investigate whether a more supportive microenvironment would further enhance the progression of reprogramming, we investigated the effects of direct Ad-CMV-MKOS injection in a clinically relevant myocardial injury model.

Myocardial infarction (MI) was induced in adult BALB/c mice by permanent ligation of the left anterior descending (LAD) coronary artery as previously described.[27] Immediately after ligation, a single intramyocardial injection of 1×10^8 IFU Ad-CMV-MKOS or Ad-CMV-Null was targeted to the border zone of the infarct region and reprogramming responses were assessed on day 6 and day 10 post MI/injection. The successful establishment of an infarct and the reproducibility of this model was confirmed by both echocardiography (Figure S3a, Supporting Information), heart weight and histological assessment of infarct size (fibrosis) using Masson's trichrome staining, which showed no significant differences between treatment groups (Figure S3b–d, Supporting Information). The efficient overexpression of OSKM by intramyocardial injection of Ad-CMV-MKOS was not impaired by the infarct as evidenced by a fold increase in *total Oct3/4* comparable to that observed previously in healthy mice (**Figure 3**a). Effective transduction was further confirmed by the presence of SOX2 positive myocytes and non-myocytes within the injected infarct border zone, which again was not different to that observed in healthy mice injected with the same dose of Ad-CMV-MKOS (Figure 3b,c). To investigate the effects of OSKM expression in the injured heart we first assessed the expression of reprogramming related and endogenous pluripotency genes that were changed in the previous experiments in healthy mice (Figure 2b). As observed in healthy animals, significant upregulation of *endogenous Oct3/4* was identified at day 6 post injection which decreased thereafter, consistent with a transient reprogramming effect (Figure 3d). Furthermore, in contrast to reports suggesting that injury promotes the efficiency and progression of in vivo reprogramming, no significant upregulation of reprogramming markers *Cdh1, Fut4* or the pluripotency related genes *Gdf3* and *Nanog* were observed. Notably, significant downregulation of *Nanog* and *Fut4* occurred on day 10 post MI/injection which further indicates a potential negative regulation of reprogramming to pluripotency in the heart (Figure 3d). Overall, this suggests that, even in the presence of injury, a single administration of Ad-CMV-MKOS induces partial reprogramming responses in cardiac tissue without the establishment of a complete pluripotency gene network. However, it remains to be determined whether alternative administration protocols offering prolonged or cyclic reprogramming factor expression could enable the further progression of reprogramming in the myocardium following a cardiac injury.

Transient in vivo reprogramming has been previously demonstrated to induce proliferation and enhance the replicative capacity of cells in various tissues including skeletal muscle,

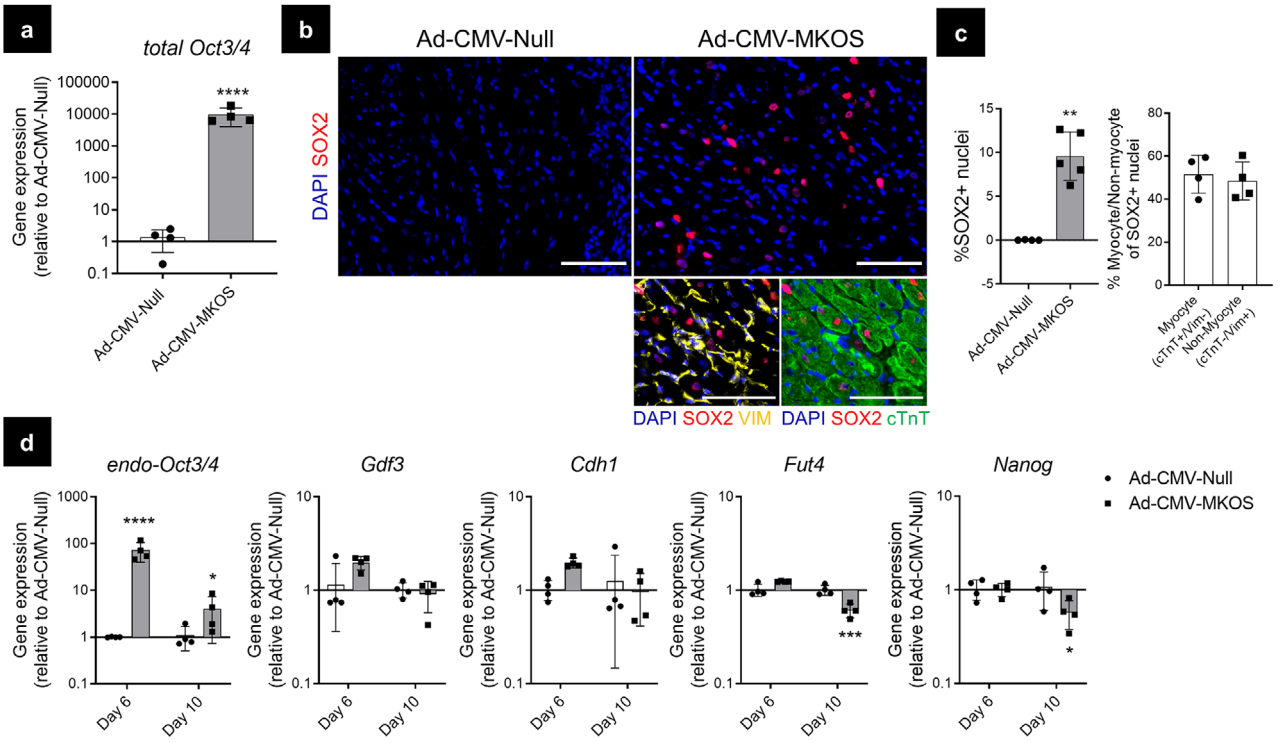

Figure 3. Adenoviral vector mediated partial reprogramming after MI. a) Relative *Oct3/4* gene expression in the left ventricle 6 days after intramyocardial injection of Ad-CMV-Null ($n = 4$) or Ad-CMV-MKOS ($n = 4$). b) Expression of SOX2 protein in the border zone of infarct hearts 3 days after injection of Ad-CMV-Null or Ad-CMV-MKOS (Scale bar = 100 μm). c) Quantification of these SOX2+ nuclei in border zone of infarct mice and percentage which are myocytes or non-myocytes ($n = 4$–5 replicates, 6 fields per replicate). d) Relative expression of endogenous pluripotency and reprogramming associated genes in the left ventricle 6 and 10 days after MI and intramyocardial injection of Ad-CMV-Null ($n = 4$) or Ad-CMV-MKOS ($n = 4$). Data presented as mean ± S.D. Unpaired *t*-test (a), Unpaired *t*-test with Welch's correction (c), Two-way ANOVA with Bonferroni correction (d), *, **, ***, **** denote $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively.

pancreas and brain.[8,9,13,14] We have previously reported that transient OSKM expression can increase the cell cycle activity of postnatal cardiomyocytes in vitro using the same adenoviral vector investigated here.[28] We therefore next explored whether OSKM expression and the induction of partial reprogramming had any downstream effects on the cell cycle activity of adult cardiomyocytes in vivo. We utilized Ki67 staining to identify cells actively in the cell cycle (G1-M) within the injected infarct border zone on days 3 and 6 post MI/injection (**Figure 4**a). Neither the percentage of Ki67+ nuclei or the number of Ki67+ myocytes (cTnT+/Vim− cells) or non-myocytes (cTnT−/Vim+ cells) appeared to be significantly different between Ad-CMV-MKOS or control vector injected hearts (Figure 4a,b). In contrast, we identified a significant increase in negative regulators of the cell cycle (*Cdkn2a/Arf*, Cdkn2a/*Ink4a*) which could be preventing the induction of cell reprogramming translating to a proliferative response (Figure 4c). Consistent with the limited effects on cardiomyocyte proliferation, no significant improvement in cardiac structure or function was identified at the latest timepoint of investigation (day 10 post MI) (Figure S4a,b, Supporting Information). Overall, these data suggest that while Ad-CMV-MKOS can facilitate the induction of partial reprogramming in the infarcted heart, this strategy, under the specific experimental conditions tested in our study, does not appear to be sufficient to generate a proliferative response that improves the regeneration of the tissue after MI. This suggests that additional barriers are present that would need to be overcome for ectopic OSKM to enhance the proliferative capacity of adult cardiomyocytes in vivo. Further investigation into the factors and pathways that regulate cardiac reprogramming both with this adenoviral system and alternative OSKM induction strategies are warranted as this could provide insight into the regulation of differentiation and cell-cycle arrest of adult cardiac cells.

3. Discussion

Transient in vivo reprogramming toward pluripotency has been demonstrated in a number of distinct tissues and is increasingly recognized as a potential strategy for tissue regeneration and rejuvenation.[29–31] However, the effects of ectopic expression of pluripotency reprogramming factors in the heart had not yet been explored. Here, we demonstrate that forced expression of OSKM mediated by direct injection of an adenoviral vector induces temporary transcriptional responses consistent with the induction of partial cell reprogramming. However, in contrast to the level of reprogramming reported within other tissues,[4,8] transient OSKM expression in the heart did not induce upregulation of the pluripotency master regulator *Nanog*. Indeed, others have also reported that time-limited OSKM in vivo leads to partial reprogramming with the absence of *Nanog* expression.[11,13]

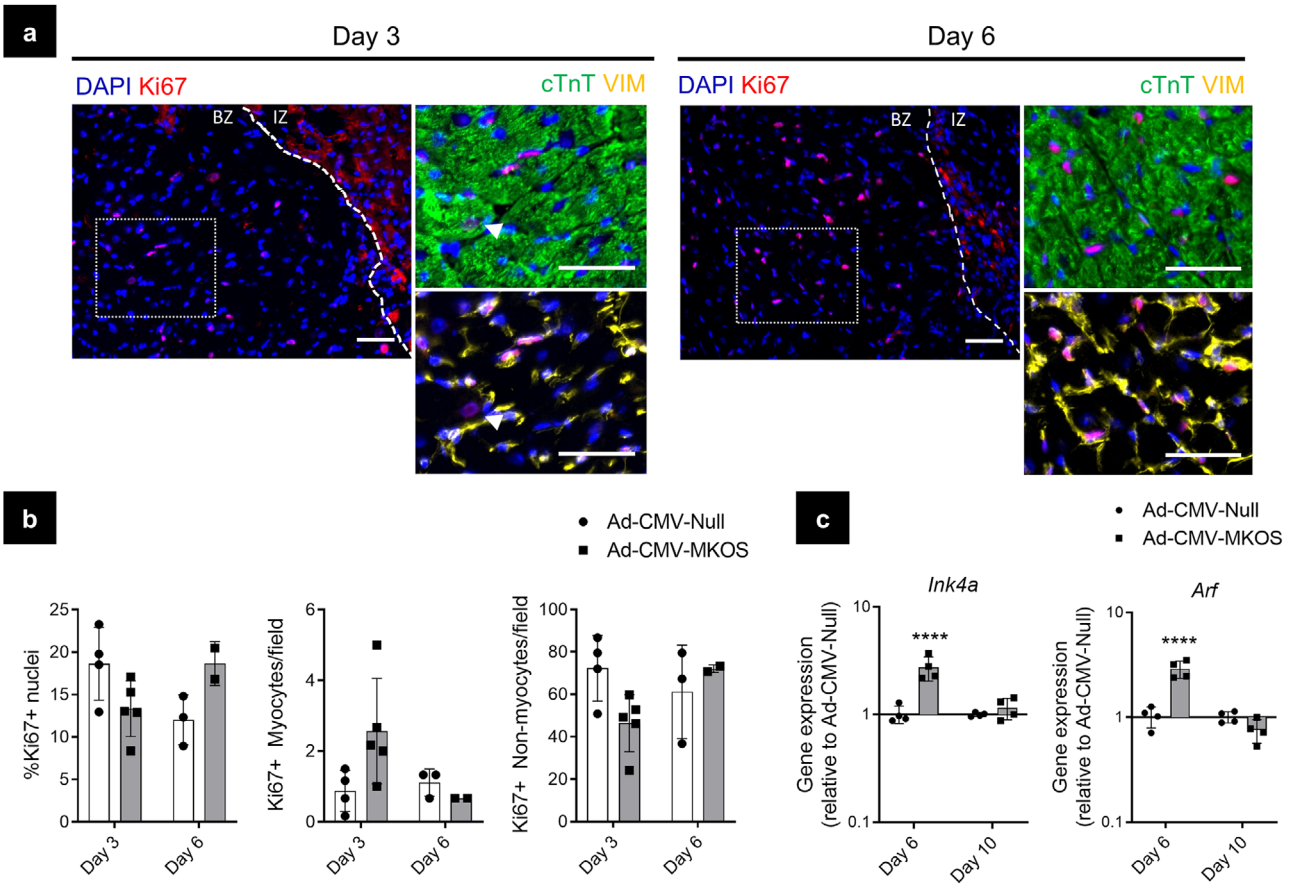

Figure 4. OSKM overexpression does not increase cardiomyocyte proliferation. a) Representative fluorescence images of Ki67 expression and co-localization within cTnT+ (white arrows) or VIM+ cells on days 3 and 6 after intramyocardial injection of Ad-CMV-MKOS (n = 2–4) BZ = border zone, IZ = infarct zone (Scale bars = 50 μm). b) Quantification of %Ki67+ nuclei and mean number of Ki67+ myocytes (cTnT+VIM−) and non-myocytes (cTnT−VIM+) per 0.15 mm^2 field (n = 2–5 replicates, 6 fields per replicate). c) Relative expression of senescence associated *Cdkn2a* (*Ink4a* and *Arf*) in the left ventricle on day 6 and 10 post MI/injection with Ad-CMV-MKOS (n = 4). Data presented as mean ± S.D. Two-way ANOVA with Bonferroni's correction (b,c), **** denotes $p < 0.0001$.

Partial reprogramming could offer benefits when considering in vivo applications of OSKM factors, given the incomplete loss of cell identity and thus less associated risk of induced tumorigenesis. Indeed, no teratomas were identified at the gross tissue level during necropsy in any experimental mice injected with Ad-CMV-MKOS in this study.

One caveat of our study is that the absence of lineage tracing tools does not allow the identification of the specific cardiac cell types that are responsible for the observed partial reprogramming responses. Indeed, this is a key challenge in the in vivo reprogramming field as a whole and the responding cell types within each tissue have remained elusive.[2,4,8,9,11,13,14] The development and application of new lineage tracing tools will be essential for future investigations to identify both the source of pluripotency gene expression changes and to understand the differentiation state, behavior and fate of partially reprogrammed cells over time.

While reprogramming appeared to be transient, we could readily detect *Oct3/4* and *Sox2* mRNA from the vector long after the downregulation of endogenous genes, evidencing that at least a proportion of transduced cells are still present in the tissue. The transiency of the response is likely due to the more rapid silencing of OSKM proteins, of which similar kinetics have been described previously for adenoviral delivery of highly regulated genes to the heart.[32] Further control of reprogramming factor expression kinetics could be achieved by inclusion of regulatable promoters within OSKM delivery vectors. The development of a doxycycline regulatable helper-dependent adenovirus encoding all four factors has recently been reported, albeit the reprogramming capabilities of this vector have not yet been described.[33] Another approach which has shown promise for reprogramming mediated cellular rejuvenation is the use of a doxycycline inducible polycistronic adeno-associated viral (AAV) vector encoding OSK which could offer further advantages regarding reduced immunogenicity.[12] However, for the safe clinical use of this vector it will be important to confirm that the high prevalence of random AAV integration previously reported for AAV delivery of OSKM factors is not also observed with this approach.[21] Moreover, the limited capacity of AAV vectors prevents delivery of the reprogramming factors and the tetracycline transactivator protein within a single polycistronic vector.[12] Indeed, adenoviral vectors offer a significant advantage given their limited

integration, increased transgene capacity and transient expression without the need for additional pharmacological control. However, a caveat of viral vectors in general that must be taken into account is that the number of reprogrammed cells may not be sufficient to result in a significant regenerative effect (in the particular case of our study, myocardial regeneration after MI), particularly in comparison to transgenic mouse models in which the reprogramming factors are encoded in the genome of the majority of cells.[2,13] In our study, we calculated that an average of 11.8% cells in the myocardium were transduced by the adenoviral vector. Given that the reprogramming process has a notoriously low efficiency, it is expected that the percentage of cells that undergo reprogramming is lower. Engineering safe and efficient vectors to deliver OKSM and replace the current transgenic models used for proof-of-principle studies is definitely one of the areas that will require most attention in the near future to advance in vivo reprogramming as a tissue regeneration or rejuvenation strategy.

The partial nature of the cardiac reprogramming response reported here was not enhanced in the presence of tissue injury, contrary to what has been reported by others.[8,18,19,26] However, the timing of OSKM overexpression in line with the release of injury associated inflammatory mediators such as IL6, which is a known key player in the enhancement of reprogramming responses, may be important.[19] We previously identified that direct intramuscular injection of plasmid DNA encoding OSKM 7 days after a severe laceration injury further induced *Nanog* compared to injection at earlier timepoints post injury.[8] While in the present study we were limited technically to inject at the time of infarct (LAD surgical ligation), future investigations could employ an earlier adenoviral injection such that the OSKM peak coincides with the early MI induced cytokine response (day 1)[34,35] or injection in the later chronic remodeling phase of disease which may provide further insight into the effects of injury on reprogramming. Moreover, the further downregulation of pluripotency related genes following the initial reprogramming response identified in both healthy and injured myocardial tissue indicates the potential existence of negative regulatory mechanisms to counteract OSKM mediated reprogramming. Further investigations into the mechanisms and pathways involved in this regulation are warranted to understand the specific roadblocks to reprogramming of cardiac cells which may further inform strategies for the manipulation of differentiation in these cell types.

4. Conclusion

Here, it is demonstrated that overexpression of OSKM reprogramming factors locally in the heart using direct injection of an adenoviral vector can initiate partial reprogramming of cardiac cells. The transient nature of this reprogramming response demonstrates the previously unreported capacity of non-integrating adenoviral vectors to enable tumor-free in vivo reprogramming. This approach could therefore offer a novel tool to further explore the outcomes of partial reprogramming in the heart. However, this strategy was unable to mount an effective regenerative response in the injured myocardium, under the experimental conditions tested. Further optimization of OSKM delivery vectors and the administration methods utilized will be important to improve the efficiency of this approach.

5. Experimental Section

Viral Vectors: Adenoviral vectors (human adenovirus type 5 (Ad5) E1/E3 deleted) were purchased from Vector Biolabs (USA). Ad-CMV-MKOS (cat no. 1789) contains cMyc-F2A-Klf4-T2A-Oct3/4-E2A-Sox2 under the control of a cytomegalovirus (CMV) promoter. The control vector Ad-CMV-Null (cat no. 1300) contains only the CMV promoter with no further transgene sequence. Vectors were supplied as in vivo grade stocks purified through sequential cesium chloride (CsCl) gradients.

Animals: Intramyocardial injections in uninjured mice were performed at the University of Manchester, UK according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and in accordance with the Animals (Scientific Procedures) Act 1986 (UK) with both local ethical and Home Office (UK) approval. Surgical procedures to model MI in mice were performed at the Cardiovascular Physiology Core at Brigham and Women's Hospital, USA and adhered strictly to the NIH Guide for the Care and Use of Laboratory Animals as well as the approved guidelines of the Institutional Animal Care and Use Committee (IACUC) at Brigham and Women's Hospital and Harvard Medical School. These experiments were performed in accordance with procedure as outlined in the BWH protocol 2016N2000266. Animals were housed in groups within ventilated cages with ad libitum access to food and water. Externally purchased animals were allowed to acclimatize to the facility for at least one week prior to any procedure.

Intramyocardial Injection: Male BALB/c (Envigo, UK) mice at 7–8 weeks of age were anesthetized by intraperitoneal (i.p.) injection of a mixture of 100 mg kg^{-1} ketamine (Narketan, Vetoquinol, UK) and 10 mg kg^{-1} xylazine (Rompun, Bayer Healthcare, UK). Mice were then intubated and connected to an external MiniVent 845 respirator (Harvard Apparatus, Germany). A constant respiratory rate of 200 breaths per minute (bpm) at 0.150 mL tidal volume was maintained throughout the surgery. A thoracotomy was performed through the 4th intercostal space to visualize the heart and enable disruption of the pericardium. Intramyocardial injection of 15 µL of adenoviral vector diluted in 0.9% saline was performed using a 30 G, 0.3 mL InsuLight syringe (Rays, Italy). Injections were targeted to the anterior/ventral wall of the left ventricle and successful injection was confirmed by a temporary pallor downstream of the needle tip. Following injection, a 5-0 prolene suture (Ethicon, UK) was used to close the ribcage and subsequently the skin. After surgery, mice were administered with 0.1 mg kg^{-1} buprenorphine (Buprenex, Reckitt Benckiser, UK) and 0.3 mL 0.9% saline via i.p. injection and allowed to recover in clean cages within a heated environment before being returned to normal housing.

Myocardial Infarction Model: MI was induced in 8 week old male BALB/c mice by permanent ligation of the left anterior descending (LAD) coronary artery as previously described[27] with some modifications. Animals were anesthetized with 3% isoflurane, intubated via the trachea and connected to a MiniVent 845 respirator with the same ventilation settings as described above. The local anesthetic lidocaine was administered at 0.1 mg kg^{-1} by subcutaneous injection immediately prior to thoracotomy. The pericardium was disrupted and an 8-0 prolene suture was utilized to ligate the LAD coronary artery. Adenoviral vectors were then administered at 1×10^8 IFU diluted in 15 µL 0.9% saline by a single direct intramyocardial injection using a 30 G, 0.3 mL InsuLight syringe targeted to the border zone of the infarct. After surgery, 0.5 mL 0.9% saline was administered, and animals were allowed to recover in clean cages in a warm environment with access to wet food. Buprenorphine (0.05 mg kg^{-1}) was administered every 12 h for 3 days alternating with carprofen (3 mg kg^{-1}) daily for up to 2 days. At the endpoints of MI experiments, tissues were extracted, and all subsequent analysis was conducted at The University of Manchester, UK.

Echocardiography: For assessment of cardiac function following MI and adenoviral injection, echocardiography was conducted and analyzed by highly experienced researchers at Brigham and Women's hospital

(Boston, USA) who were blinded to the treatment groups. Conscious echocardiography was performed on a Vevo 3100 Imaging system (Fujifilm, VisualSonics, Canada). Left ventricular internal dimension at diastole and systole (LVIDd/s), left ventricular mass (LV mass), heart rate, ejection fraction, fractional shortening and fractional area change were calculated using Vevolab (Fujifilm, VisualSonics) Imaging Software.[36]

Gene Expression Analysis: Animals were culled by cervical dislocation, detailed necropsy was performed and hearts were excised, washed in ice-cold PBS prior to dissection of the atria and isolation of anterior/ventral portion of the ventricles for RNA analysis. Tissue samples were then homogenized in TRIzol reagent (Life Technologies, UK) using the TissueLyser LT (Qiagen, UK). Homogenate was then subjected to chloroform phase separation and RNA was then extracted from the colorless aqueous phase using the PureLink RNA Mini kit with On-Column DNase (Invitrogen, UK) treatment following the manufacturer's guidelines. RNA was eluted in 50 µL RNase free water and further DNase treatment using the RapidOut DNA removal kit (ThermoFisher, UK) was conducted following manufacturer's guidelines. RNA was quantified by absorption at 260 nm (A260) using a BioPhotometer (Eppendorf, UK). cDNA was synthesized from 1 µg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) following manufacturer's instructions. cDNA samples (2 µL) were combined with PowerUp SYBR Green Mastermix (ThermoFisher) with forward and reverse primers (500 nM) following manufacturer's guidelines. RT-qPCR reactions were run in duplicate on a CFX96 thermal cycler which included melt curve analysis to ensure amplification of a single PCR product at the expected dissociation temperature. Non-reverse transcribed controls (NRTC) were used to confirm no contamination with viral or genomic DNA. High throughput integrated microfluidic RT-qPCR was also carried out using a Biomark HD (Fluidigm, UK) with the 96 × 96 dynamic array. Data were analyzed using the Livak method ($2^{-\Delta\Delta Ct}$)[37] using β-actin, or the geometric mean of Rps13 and Mapk1 as housekeeping genes and comparing to the relevant controls for each experiment to calculate relative (fold-change) gene expression. Primer pairs used for RT-qPCR are provided in Table S5 (Supporting Information).

Immunofluorescence: Animals were culled under terminal anesthesia by intracardiac injection of 30×10^{-3} M potassium chloride (KCl) followed by cervical dislocation. Necropsy was then performed to visualize all major organs to determine the presence or absence of gross tissue abnormalities such as tumors. The heart was extracted and washed in sterile KCl before being cut transversely and embedded in optimal cutting temperature (OCT) solution (Pioneer Research Chemicals, UK) and frozen by immersion in isopentane pre-cooled in liquid nitrogen. 8–10 µm transverse cryosections through the whole heart were taken with a Leica CM3050S cryostat (Leica, UK). Cryosections were fixed in either ice-cold acetone or pre-cooled 4% paraformaldehyde for 10 min at room temperature. Slides were then washed twice in PBS before permeabilization with 0.3% Triton-X in PBS for 10 min at room temperature. Three further washing steps in PBS-0.1% Triton-X (PBS-T) for 5 min each were conducted before sections were incubated with PBS-T containing 1% BSA (Sigma-Aldrich, UK) + 0.3 M glycine for 30 min at room temperature. Blocking was then continued by incubation in PBS-T containing 10% normal goat serum (NGS) + 0.3 M glycine for an additional 1 h at room temperature. Following blocking, sections were incubated with primary antibodies diluted in PBS-T containing 10% NGS overnight in a humidified chamber at 4 °C. The following day slides were washed five times in PBS-T for 10 min each before being incubated with secondary antibodies diluted in PBS-T containing 10% NGS for 1 h at room temperature in the absence of light. Slides were then washed three times in PBS-T for 10 min each, twice in PBS for 5 min each and rinsed for 30 s in dH_2O. After air drying, slides were then mounted with ProLong Gold Antifade with DAPI (Invitrogen) which was allowed to cure overnight at room temperature before imaging. Fluorescence microscopy images were acquired with a Zeiss AXIO Observer. A1 microscope using a 20× or 40× objective. A minimum of 4 random fields of view were acquired for each biological replicate unless otherwise stated in the figure legend. Images were analyzed and quantified manually using the cell counting feature on ImageJ (NIH, USA). To identify myocytes from non-myocytes the cell specific markers cardiac troponinT (cTnT) for myocytes and vimentin (Vim) for non-myocytes were utilized as previously described.[38] The antibodies used in these investigations are given in Table S3 (Supporting Information).

Statistical Analysis: A minimum of 3 biological replicates was included for each experiment unless otherwise stated in the figure legend. Statistical analysis was conducted using mean values from each biological replicate and did not recognize technical replicates (fields of view/duplicate PCR reactions) as individual *n*-numbers. For RT-qPCR data statistical analysis was conducted on untransformed ΔCt values. Statistical analysis was carried out using GraphPad Prism 8 (GraphPad, USA) to perform unpaired Student's *t*-tests for comparisons between 2 groups with Welch's correction applied when unequal variance was identified. For comparisons between 3 or more groups one-way or two-way ANOVA followed by Bonferroni's correction or Tukey's post-hoc analysis was utilized. A probability (p) of < 0.05 was regarded as statistically significant and p values and n numbers are specified in the figure legends. All data are presented as mean ± standard deviation (S.D.).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Author contributions

T.K., I.d.L., and K.K. conceived the project and designed experiments. T.K. and I.d.L. performed the majority of experiments. S.F. performed echocardiography and advised in experimental design. E.J.C. and G.C. advised in experimental design and interpretation of data. T.K., I.d.L., S.F., G.C., and K.K. wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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