Full title: The calcineurin variant CnAβ1 improves post-infarction ventricular remodelling by promoting infarct vascularisation

Running title: CnAβ1 promotes infarct vascularisation

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ABSTRACT

Aims – Ventricular remodelling following myocardial infarction progressively leads to loss of contractile capacity and heart failure. Although calcineurin promotes maladaptive cardiac hypertrophy, we recently showed that the calcineurin splicing variant CnAβ1 has beneficial effects on the infarcted heart. However, whether this variant limits necrosis or improves remodelling is still unknown, precluding translation to the clinical arena. Here we explored the effects and therapeutic potential of CnAβ1 overexpression post-infarction. *Methods and Results* - Double transgenic mice with inducible cardiomyocyte-specific overexpression of CnAβ1 underwent left coronary artery ligation followed by reperfusion. Echocardiographic analysis showed depressed cardiac function in all infarcted mice 3 days post-infarction. Induction of CnAβ1 overexpression one week after infarction improved function and reduced ventricular dilatation. CnAβ1-overexpressing mice showed shorter, thicker scars and reduced infarct expansion, accompanied by reduced myocardial remodelling. CnAβ1 induced vascular endothelial growth factor (VEGF) expression in cardiomyocytes, which resulted in increased infarct vascularisation. This paracrine angiogenic effect of CnAβ1 was mediated by activation of the Akt/mTOR pathway and VEGF. *Conclusions* - Our results indicate that CnAβ1 exerts beneficial effects on the infarcted heart by promoting infarct vascularisation and preventing infarct expansion. These findings highlight the translational potential of CnAβ1 for gene-based therapies.

Keywords: CnAβ1, calcineurin, myocardial infarction, cardiac remodelling, Akt

Myocardial infarction remains a major cause of mortality and hospitalisation worldwide. Reperfusion of the blocked artery through percutaneous coronary intervention or thrombolytic therapy reduces infarct size and increases survival[1]. However, even if reperfusion therapies have allowed a dramatic reduction in immediate mortality, infarction survivors are at high risk of suffering from heart failure, arrhythmia and sudden death. Shortly after reperfusion millions of cardiomyocytes die due to oxidative stress and are substituted by scar tissue. The mechanical stress experienced by the ventricle progressively causes scar thinning and expansion, leading to dilatation of the left ventricle and other structural changes collectively known as remodelling[2, 3]. The surviving cardiomyocytes undergo hypertrophy to compensate the loss of contractile capacity, but these changes progressively lead to a decline in cardiac function and eventually to the development of heart failure.

Far from being a passive tissue, post-infarction scar tissue is populated by different cell types that contribute to maintaining its integrity, regulate extracellular matrix (ECM) turnover and prevent dilatation[2-4]. A few days after infarction a granulation tissue is formed, integrated by leukocytes, new blood vessels, fibroblasts and myofibroblasts that secrete different ECM components, mainly collagen[5]. Inflammatory cells progressively disappear from the area and the scar matures into an ECM-rich fibrotic tissue[6]. The persistence of myofibroblasts in the infarct region contributes to tissue stability. Progressive loss of these cells causes thinning of the infarcted area and left ventricular dilatation.

Whereas a strong ECM in the scar region prevents structural remodelling, collagen accumulation in the remote myocardium has the opposite effect. Interstitial fibrosis increases

passive stiffness, causes electrical remodelling and enhances arrhythmogenicity, further contributing to cardiac remodelling and dysfunction[2]. Therefore, ideal therapies will aim at promoting scar maturation while reducing interstitial fibrosis in the remote myocardium.

The molecular mechanisms involved in post-infarction remodelling are not entirely understood[7]. Calcineurin generally plays a detrimental role in the heart and both protective and deleterious effects have been described for this protein in the response to ischemia/reperfusion[8-10]. It is both sufficient and necessary to induce maladaptive cardiac hypertrophy[11, 12], and activation of the transcription factor NFAT by calcineurin promotes myocardial remodelling and chamber dilatation following infarction[13]. We recently described that, in contrast to other calcineurin A isoforms, the naturally-occurring splicing variant CnAβ1 has a beneficial effect on the heart. CnAβ1 has a unique C-terminal domain, not present in any other known protein, that confers it distinct properties[14]. In a chronic myocardial infarction model with permanent occlusion of the left coronary artery, cardiac-specific overexpression of CnAβ1 has a protective effect, improving cardiac function and reducing long-term scar size[15].

The purpose of the present work was to investigate the potential benefit of CnAβ1 overexpression in response to ischemia/reperfusion injury, to determine whether CnAβ1 is cardioprotective or whether its beneficial action results from post-infarction effects (i.e. improving post-infarction remodelling), and to explore the potential of CnAβ1 for gene-based therapies.

METHODS

Transgenic Mice

rtTA-CnAβ1 mice express the reverse Tet transactivator (rtTA) in a cardiomyocyte-specific manner[16], which in turn induces CnAβ1 overexpression from a second transgene in a doxycycline-inducible fashion (Fig. 1). rtTA mice also express rtTA in a cardiomyocyte-specific manner but lack the CnAβ1 transgene and therefore don't overexpress CnAβ1. The rtTA-CnAβ1 mouse line was generated by crossing the original TetO-CnAβ1 transgenic line, carrying the Tet operator and the CnAβ1 cDNA (Fig. 1) with the rtTA mouse line. Both rtTA-CnAβ1 and rtTA mice were generated in a CBA/Black10 genetic background[16] and inbred in this background for at least eight generations. Male littermates between 3-5 months of age were used for experiments. Doxycycline (Dox) was administered to the mice with the diet (0.3%) starting either 3 weeks before infarction or 1 week after infarction, and maintained until mice were sacrificed.

Surgeries and Echocardiographic analysis

Myocardial infarction was induced in rtTA-CnAβ1 (n=28) and rtTA mice (n=21) by ligation of the left coronary artery for 30 min followed by reperfusion of the artery. Surgeries were carried out under mechanical ventilation with 3-3.5% sevoflurane. Mice received analgesic treatment with buprenorphine (0.3 mg/kg s.c.) after surgery. The mortality rate in the first 24 h post-infarction was 38%; no mortality was found afterwards. Cardiac function, chamber dilatation and wall thickness were analysed by transthoracic echocardiography 3 and 28 days after infarction, as well as in uninfarcted mice, using a Vevo 2100 system and a 45 MHz probe (Visualsonics, Toronto, Canada). Measurements were carried out by a blinded operator

with mice placed on a heating pad under light anaesthesia with sevoflurane adjusted to obtain a target heart rate of 500 ±50 beats per minute[15]. Two-dimensional (2D) and M-mode echocardiography images were recorded in long and short view at the level of the papillary muscles. Left ventricle (LV) end-systolic, end-diastolic volumes and LV ejection fraction were measured from 2D parasternal long axis using the area-length method. Animals were sacrificed by gradually filling the chamber with carbon dioxide.

For the analysis of infarct size using echocardiography, regional left ventricular function was evaluated in the parasternal long-axis view. The left ventricle wall was subdivided into six segments (basal, mid and apical in the anterior and posterior walls). Each segment was scored by an independent blinded evaluator based of its motion and systolic thickening, according to the guidelines of the American Society of Echocardiography[17] (1, normal or hyperkinetic; 2, hypokinetic; 3, akinetic, negligible thickening; 4, dyskinetic, paradoxical systolic motion; 5, aneurysmal, diastolic deformation). The number of dysfunctional segments was quantified and the total score representing the sum of the score of the six individual segments in each heart was calculated.

All experiments were approved by the local Ethics Committee at the Centro Nacional de Investigaciones Cardiovasculares. The investigation conforms to the principles of Laboratory Animal Care which are formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication 85-23, 1996).

Western Blot

Western blot was carried out as previously described[18] using the following primary antibodies: anti-phospho-Akt-Ser473, anti-Akt, anti-phospho-mTOR-Ser2448, anti-mTOR (Cell Signaling), anti-HIF1α and anti-Periostin (Novus Biochemicals), anti-VEGF, anti-Lox and anti-CD31 (Abcam), anti-fibronectin (Sigma). Anti-CnAβ1 has been previously described[14]. Brightness and contrast were linearly adjusted using Photoshop CS5.

Histology and Immunohistochemistry

Samples were fixed in paraformaldehyde (4% in PBS) for 48 h, washed in PBS, dehydrated and included in paraffin. Five microns-thick sections were stained following Masson's trichrome protocol[14]. Images were quantified using ImageJ (NIH, USA). Scar length was determined in Masson's trichrome-stained sections using the midline method, which best correlates with functional measurements[19]. In this method, the infarct length is measured as the length of the midline of the infarcted wall in which >50% of the wall thickness is composed of scar tissue[19]. Scar length represents the percentage of infarct length with respect to the length of the whole LV circumference. Medium/large blood vessels in the infarct region were visually scored in Masson trichrome-stained sections. These vessels would be >20 μm in diameter and typically surrounded by a smooth muscle layer that makes them easily identifiable. In parallel, vessels were stained by immunohistochemistry using anti-αSMA. In both experiments, the number of vessels was determined in the infarct region only and divided by the infarct area. Vessels were quantified in three non-consecutive sections for each mouse. Capillaries were stained using biotin-conjugated Isolectin B4 (Sigma) and scored in two separate 40x microscope fields. Apoptosis in cardiomyocytes (in the whole section) and fibroblasts (in the infarct region) was analysed using Tunel and immunostaining with anti-troponin I (cardiomyocytes) or anti-periostin (fibroblasts).

RNA isolation and qRT-PCR

After sacrificing the animals, mice were perfused with PBS, hearts were excised and samples from the infarct region, border zone and remote myocardium were separated and snap-frozen in liquid nitrogen. Total RNA was isolated using the RNeasy kit from Qiagen, with DNAse digestion on the column. cDNA was synthesized from 100 ng of total RNA using random hexamers and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a 10 μl reaction. Quantitative reverse-transcribed polymerase chain reaction (qRT-PCR) was carried out in an AB9700 thermocycler (Applied Biosystems) using Taqman chemistry or SYBR green (Applied Biosystems). The following Taqman probes were used: Acta1 (Mm00808218_g1), Nppb/BNP (Mm01255770_g1), Col1a1 (Mm00801666_g1), Lox (Mm00495386_m1) Thy1 (Mm00493681_m1), and Acta2 (Mm01204962_gh). Gene expression was normalised to 18S rRNA levels quantified simultaneously using a VIC-labelled probe (4310893E, Applied Biosystems). CnAβ1 and CD31 expression were quantified using SYBR green and the following primers and conditions: CnAβ1 forward: 5'-AGAAGGTGAAGACCAGT-3', CnAβ1 reverse: 5'-AGCAAGTTGCATAACATCATT-3', CD31 forward: AATGGCAACTGGAGCGAGCACT, CD31 reverse: GGAGAAGGCGAGGAGGGTTAGGT; 2 min at 50° C, 10 min at 95° C and 40 cycles of 15 sec at 95° C, 30 sec at 60°. CnAβ2 primers were previously described[15]. qRT-PCR data was analysed using LinReg software in order to estimate the efficiency rates and the Ct values[20].

Angiotubes

Neonatal cardiomyocytes were isolated from rtTA-CnAβ1 and rtTA mice as described[21]. Neonatal mice were sacrificed by cervical dislocation. A total of 750.000 cells/well were seeded in 12-well plates and grown in the presence of 10% fetal calf serum (FCS) for 2 days. Cells were then washed and cultured in serum-free medium for 2 days in the presence of 2 μg/ml doxycycline, 0.1 mM rapamycin or DMSO (1:1000) as indicated in the figures. Conditioned medium was cleared by centrifugation and used for angiotube assays as follows. Matrigel (Becton Dickinson) diluted 1:3 in DMEM was added to 96-well plates (100 μl/well) and allowed to polymerise for 60 min. Human umbilical cord vascular endothelial cells (HUVEC, Promocell)[22] were seeded at a density of 30.000 cells/well in the presence of the different conditioned mediums and allowed to form tubes for 6 h. Where indicated, neutralising anti-VEGF antibody (R&D Systems) was added to the culture (2 ng/μl). Anti-troponin I (Abcam) was used as a negative control antibody. Pictures were taken with a Nikon eclipse TI microscope and the number of tube network nodes was quantified for each well. Experiments were carried out in triplicate and all experiments were repeated at least three times.

Statistics

Data are presented as mean ±SE. In echocardiographic data, the same mice were analysed 3 days and 28 days post-infarction, with non-infarcted animals representing a different group of mice. To test for statistical significance, data were analysed by 2-way ANOVA followed by Bonferroni post-test for multiple comparisons. In addition, a 2-way ANOVA with repeated measures followed by Bonferroni post-test was applied to compare mice at 3 days vs. 28 days post-infarction. Group differences in qRT-PCR and histological quantifications were analysed by 1-way ANOVA followed by Dunnett's post-test to compare to Dox-untreated

mice. Angiotubes were analysed by student's t-test or 2-way ANOVA followed by Bonferroni's post-test depending on the number of variables. Data were analysed with GraphPad Prism 5.0 (Graphpad Software Inc., www.graphpad.com) and differences were considered statistically significant at $p<0.05$.

RESULTS

CnAβ1 improves cardiac function and remodelling after ischemia/reperfusion

To determine the potential benefit of CnAβ1 overexpression in the context of myocardial infarction with reperfusion, we developed double transgenic mice in which CnAβ1 expression is induced 3-4-fold specifically in cardiomyocytes upon administration of Dox in the diet (rtTA-CnAβ1 mice, Fig. 1). As negative controls, to test the effect of Dox itself, we used mice that overexpress the same rtTA transactivator but lack the CnAβ1 transgene (rtTA mice). As a first approach we administered Dox starting 3 weeks before surgery and maintained it throughout the experiment. We induced myocardial infarction by occluding the left coronary artery for 30 min. followed by reperfusion, and analysed the mice 3 and 28 days later by echocardiography. rtTA-CnAβ1 and rtTA mice showed functional decline and chamber dilatation 3 days post-infarction regardless of Dox treatment (Fig. 2A-D, Table 1), suggesting that initial infarct size and myocardial loss was analogous among all groups. At day 28 post-infarction we detected a significant improvement in cardiac function (left ventricular ejection fraction [LVEF]) in mice overexpressing CnAβ1 from 3 weeks before surgery (Dox Pre-MI, Fig. 2A, grey bars), while animals not receiving Dox showed no improvement (No Dox, Fig. 2A, white bars). Improved function was accompanied by reduced ventricular dilation in Dox-treated rtTA-CnAβ1 mice (Fig. 2C, Table 1). In contrast, Dox administration to rtTA mice had no effect on cardiac function or chamber dilation (Fig. 2B, 2D). Of note, neither CnAβ1 nor CnAβ2 (the CnAβ splicing isoform that carries the full CnA autoinhibitory domain) mRNA expression showed much variation in response to myocardial infarction itself (Fig. 1G).

CnAβ1 induces recovery, rather than protection

The results obtained at 3 days post-infarction suggested that CnAβ1 offered no protection against reperfusion injury whereas the improvement observed at 28 days suggested a positive effect post-infarction. To further discriminate between the protective and recovery effects of CnAβ1 overexpression, we treated rtTA-CnAβ1 mice with Dox starting one week post-infarction, once the scar has developed (Dox post-MI, black bars). Interestingly, a significant enhancement of cardiac function was observed in these mice (Fig 2A), which was accompanied by reduced ventricular dilatation (Fig. 2C). Importantly, in all the study the same mice were sequentially analysed at 3 and 28 days. The functional decline observed at 3 days post-infarction and the subsequent improvement observed at 28 days in Dox-treated rtTA-CnAβ1 mice (Fig. S1, Table 1), demonstrates that CnAβ1 induces functional recovery, rather protection from infarction.

CnAβ1 prevents infarct expansion and improves remote myocardium remodelling

We next analysed the impact that CnAβ1 overexpression has on infarct expansion. We found that overexpression of CnAβ1 before infarction resulted in reduced scar length 28 days post-infarction, compared to mice untreated with Dox (Fig. 3A, Fig. S2). Importantly, a similar effect was achieved when CnAβ1 was induced after infarction. In Dox-treated rtTA-CnAβ1 mice, scar length at 28 days post-infarction (20.47% ±3.85 in mice treated pre-MI; 14.23% ±4.16 in mice treated post-MI) remained similar to that observed at 7 days in untreated mice (21.52% ±2.90). Echocardiographic analysis showed that CnAβ1 overexpression results in a reduction in the number of left ventricle segments with dysfunctional motility 28 days after

infarction (Table S1). This was particularly evident in the mid anterior segment, confirming the reduced infarct size observed by histological methods. In addition, CnAβ1-overexpressing mice showed thicker scars than control mice (Fig. 3B), suggesting that CnAβ1 prevents infarct expansion. Induction of CnAβ1 also resulted in higher expression of collagen Iα1 in the scar region, together with increased expression of lysil oxydase, which crosslinks collagen and elastin molecules into mature fibres, the fibroblast proliferation marker Thy1/CD90 and α-smooth muscle actin (Fig. 3C-F). We also detected increased expression of periostin, fibronectin and lysil oxidase proteins in the infarct region of CnAβ1 overexpressing mice (Fig. 3G). rtTA mice showed no changes in scar length, thickness or expression of fibrosis markers upon Dox administration (Fig. 3A-G, Table S1). To investigate whether reduced infarct expansion was accompanied by improved fibroblast survival, we quantified the percentage of apoptotic fibroblasts by Tunel staining. As shown in Fig. S3A, only a very low degree of fibroblast apoptosis was detected in the infarct region 28 days post-infarction and no significant difference among the groups was observed.

To determine whether changes in infarct expansion were paralleled by improved remodelling of the remote myocardium, we analysed the cardiomyocyte area and the expression of heart failure markers in this region. CnAβ1 overexpression either before or after infarction significantly reduced the cross-sectional area of cardiomyocytes and the wall thickness in the remote myocardium (Fig. 4A, Table 1). This was accompanied by a significant reduction of α-skeletal actin, BNP and collagen Iα1 in the remote myocardium of these mice (Fig. 4B-D). A trend towards reduced cardiomyocyte apoptosis was also detected in CnAβ1-overexpressing mice, although the percentage of apoptotic cardiomyocytes 28 days post-infarction was low (Fig. S3B). In contrast, Dox administration to rtTA mice had no effect on cardiomyocyte size or expression, heart failure or fibrosis markers or apoptosis (Fig. 4A-D, Fig. S3B).

Improved vascularisation in the infarct region of CnAβ1-overexpressing mice

The effect of CnAβ1 on infarcted hearts is reminiscent of cardiomyocyte activation of the hypoxia inducible factor 1α (HIF1α), which drives expression of angiogenic factors and promotes infarct vascularisation[23]. To determine whether more active, thicker scars were supported by enhanced vascularisation in CnAβ1-overexpressing mice, we quantified the amount of blood vessels in the scar region. We observed that induction of CnAβ1 overexpression either pre- or post-infarction increased the number of blood vessels in the infarcted region (Fig. 5, Fig. 6A, 6B). This was confirmed by an upregulation of CD31 and αSMA mRNA in the same region (Fig. 6C, Fig. 3F, 3G). A mild induction in CD31 mRNA expression and in the number of capillaries was detected also in the remote area upon CnAβ1 induction (Fig. 6E, 6F).

CnAβ1 promotes vascularisation by activating the Akt signalling pathway

We have previously shown that CnAβ1 activates the Akt pathway through its unique C-terminal domain[15]. Akt is known to induce angiogenesis in the heart by promoting expression of VEGF[24]. We therefore investigated the role of this signalling pathway in the angiogenic response elicited by CnAβ1. We observed that Dox stimulation of rtTA-CnAβ1 mice induced expression of VEGF and the hypoxia inducible factor 1α (HIF1α) in the remote myocardium (Fig. 7A). This was accompanied by increased CD31 expression and activation of the Akt/mTOR pathway. No changes were observed in rtTA mice upon Dox treatment.

To test whether the paracrine angiogenic effect of CnAβ1 was reproduced in culture, we used the assay for HUVEC angiotube formation on matrigel[25]. As shown in Fig. 7B,

conditioned medium from Dox-treated rtTA-CnAβ1 cardiomyocytes stimulated angiotube formation by HUVEC, whereas that of untreated cells or rtTA cardiomyocytes had no effect. This effect was blocked by a neutralising anti-VEGF antibody (Fig. 7C), suggesting that VEGF mediates the paracrine effect of CnAβ1-overexpressing cardiomyocytes on endothelial cells. Interestingly, treatment of rtTA-CnAβ1 cardiomyocytes with the inhibitor of the Akt/mTOR pathway rapamycin blocked the induction of angiotube formation by the conditioned medium (Fig. 7D). Rapamycin also prevented the induction of VEGF secretion by Dox-treated rtTA-CnAβ1 cardiomyocytes (Fig. 7E). These results suggest that CnAβ1 activates Akt/mTOR in cardiomyocytes to induce VEGF secretion and paracrine activation of angiogenesis.

DISCUSSION

We show here that, in contrast to other calcineurin isoforms, CnAβ1 has beneficial effects on the heart after reperfusion injury. CnAβ1 reduces ventricular dilatation and improves function even when overexpressed one week after infarction. By activating the Akt/mTOR pathway, CnAβ1 promotes secretion of angiogenic mediators by cardiomyocytes and enhances vascularisation of the infarct region, thus precluding infarct expansion and improving heart remodelling.

Our results suggest that the beneficial effects of CnAβ1 overexpression involve the prevention of heart remodelling, rather than cardioprotection. The significant ventricular dilatation and decline in cardiac function observed here at day 3 post-infarction in all experimental groups strongly suggests that CnAβ1 exerts its beneficial effects by improving myocardial remodelling after infarction, rather than by protecting from reperfusion injury. This is further supported by the fact that induction of CnAβ1 as late as 1 week post-infarction improves cardiac function and reduces ventricular dilatation. The scar tissue in CnAβ1-overexpressing mice is thicker and richer in collagen, matrix crosslinking enzymes and activated fibroblasts, which results in reduced infarct expansion.

We previously showed that CnAβ1 overexpression leads to reduced scar formation after permanent occlusion of the left coronary artery[15]. In the chronic infarction model, scars from CnAβ1-overexpressing mice showed reduced fibroblast number and collagen expression. In contrast, Dox-treated rtTA-CnAβ1 show thicker scars with increased collagen expression after ischemia reperfusion. This apparent discrepancy is likely due to the different response to permanent ischemia and to reperfusion injury. Chronic myocardial infarction

eventually results in the death of most cells in the infarct region, leading to loss of extracellular matrix production and infarct expansion. In that context, the reduced infarct size observed in the presence of CnAβ1 is likely due to reduced myocardial damage, which is consistent with the improved function already observed 7 days post-infarction[15]. In contrast, reperfusion injury reduces cardiomyocyte death and promotes a stronger angiogenic response in the infarcted myocardium[26]. Our results suggest that CnAβ1 promotes VEGF expression from cardiomyocytes to improve vascularization of the infarcted region, while stimulating fibroblast proliferation and ECM production in this area. In addition, although we only detected a very low degree of fibroblast apoptosis 28 days post-infarction, we can not exclude the possibility that better vascularization improves fibroblast survival at earlier time points. The increase in fibroblasts and myofibroblasts in the scar tissue will contribute to increased extracellular matrix turnover and to the maintenance of the scar structure, thus preventing remodelling. Distinct effects on heart remodelling in response to permanent ischemia and reperfusion injury have been described for other factors like GDF15[27].

In addition, the remote myocardium of CnAβ1-overexpressing mice shows reduced cardiomyocyte hypertrophy, together with lower expression of collagen and heart failure markers. This reduction in myocardial remodelling is likely the result of the limited infarct expansion. Importantly, all these effects were observed even when CnAβ1 overexpression was induced late after infarction, indicating that CnAβ1's main target is not the reduction of cardiomyocyte death or inflammation, but the prevention of infarct expansion and chamber dilatation.

Molecular therapies capable of improving ventricular function after infarction are scarce. Overexpression of sonic hedgehog starting 5 days post-infarction attenuates remodelling and improves function[28]. Inhibition of Wnt signalling with Frizzled antagonist peptides reduces infarct expansion and prevents ventricular dilatation[29]. This effect was also

achieved when the peptide was administered two weeks after infarction, suggesting that Wnt signalling has a detrimental effect on heart remodelling rather than on early infarct healing. In both cases, the improvement in cardiac remodelling was accompanied by enhanced infarct vascularisation. In this same regard, overexpression of the angiogenesis regulator HIF1α also improves vascularisation and function, and reduces infarct size in transgenic mice[23]. However, chronic overexpression of HIF1α has detrimental effects on the heart[30]. Similarly, short-term Akt activation in cardiomyocytes induces VEGF and angiogenesis but sustained activation induces maladaptive hypertrophy and fibrosis[24]. We show here that CnAβ1 induces angiogenesis by promoting VEGF expression in an Akt/mTOR-dependent manner. In contrast to sustained Akt activation, CnAβ1 overexpression in cardiomyocytes improves infarct vascularisation, cardiac remodelling and function without inducing the detrimental side effects observed after sustained Akt or HIF1α overexpression.

Together our results underline the therapeutic potential of CnAβ1 and suggest that it may be an excellent candidate for gene therapies aimed at reducing ventricular remodelling and improving cardiac function post-infarction. We demonstrate that these beneficial effects can be achieved by inducing CnAβ1 expression late after infarction. Our work constitutes a first step for the translation of these findings to the clinical setting.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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FIGURE LEGENDS

Figure 1. Cardio-specific inducible transgenic mice used in this study. A, rtTA-CnAβ1 mice overexpress the reverse Tet transactivator (rtTA) specifically in cardiomyocytes under the control of the Xenopus MLC2v promoter. Upon doxycycline (Dox) administration in the diet, rtTA activates overexpression of CnAβ1 from a second transgene. **B**, Schematic showing the different experimental groups and Dox administration regime. **C**, CnAβ1 mRNA expression was analysed in the remote myocardium 28 days post-infarction by qRT-PCR. Results are expressed as mean fold induction ±SE over the values of uninjured hearts (indicated by a dashed line). *$p<0.05$ Dox-treated vs no Dox. **D**, rtTA mice were used as negative controls to test for the effect of rtTA overexpression and Dox administration themselves. These mice overexpress rtTA in cardiomyocytes but not CnAβ1, since they lack the second transgene present in rtTA-CnAβ1 mice. **E**, Schematic showing the rtTA mouse groups and Dox administration regime. **F**, Analysis of CnAβ1 mRNA expression in the remote myocardium 28 days post-infarction by qRT-PCR. Results are expressed as mean fold induction ±SE over the values of uninjured hearts (dashed line). n=6-15 per group. **G**, qRT-PCR analysis of CnAβ1 and CnAβ2 mRNA expression in uninjured hearts and in the remote and infarct regions of the heart 28 days post-infarction. *$p<0.05$ compared to no infarction, 1-way ANOVA plus Bonferroni post-test.

Figure 2. Induction of CnAβ1 overexpression late after ischemia/reperfusion improves cardiac function and reduces dilatation. A Tet-on inducible transgenic mouse with

cardiomyocyte-specific expression of the reverse Tet transactivator (rtTA) was used. CnAβ1 overexpression was induced in rtTA-CnAβ1 mice with doxycycline starting either 3 weeks before (Dox pre-MI) or 1 week after (Dox post-MI) ischemia/reperfusion (**A, C**). Control rtTA mice (**B, D**) lack the CnAβ1 transgene and therefore do not overexpress CnAβ1 upon doxycycline treatment. Ejection fraction (**A, B**) and left ventricular end systolic volume (**C, D**) were analysed by echocardiography 3 and 28 days post-infarction. Graphs show means ±SE. n=6-15 per group. *p<0.05 infarcted vs no MI, ***p<0.0005 infarcted vs no MI, #p<0.05 Dox-treated vs no Dox, 2-way ANOVA plus Bonferroni post-test. §p<0.05 28 days post-MI vs 3 days post-MI repeated-measures 2-way ANOVA followed by Bonferroni post-test.

Figure 3. CnAβ1 reduces infarct expansion. A, B Scar length (A) and thickness (B) were analysed 28 days post-infarction using histological methods. **C-F**, mRNA expression of collagen Iα1 (C), lysyl oxidase (D), Thy1 (E) and α-smooth muscle actin (F) were analysed by qRT-PCR in the infarct region only. Results are expressed as mean fold induction ±SE over the values of uninjured hearts (dashed line). n=6-15 per group. *p<0.05 Dox-treated vs no Dox, 1-way ANOVA followed by Dunnett's post-test. **G**, Western blot analysis of lysyl oxidase (Lox), fibronectin (Fn), periostin (Postn) and CD31 in the infarct region of rtTA-CnAβ1 and rtTA mice 28 days post-infarction.

Figure 4. Reduced myocardial remodelling in CnAβ1-overexpressing mice. A, Cardiomyocyte cross-sectional area was analysed 28 days post-infarction by histological

methods. **B-D**, The expression of α-skeletal actin (B), BNP (C) and collagen Iα1 (D) mRNA was analysed by qRT-PCR in the remote myocardium. Results are expressed as mean fold induction ±SE over the values of uninjured hearts (dashed line). n=6-15 per group. *p<0.05 Dox-treated vs no Dox, 1-way ANOVA followed by Dunnett's post-test.

Figure 5. Reduced infarct expansion in CnAβ1-overexpressing mice is accompanied by improved infarct vascularisation. rtTA-CnAβ1 and rtTA mice, either treated or untreated with doxycycline, were sacrificed 28 days post-infarction. Hearts were excised, fixed and stained with Masson's trichrome protocol (left and centre) or with anti-αSMA and DAPI (right). Histological sections of the infarct region show an increase in the number of medium and large vessels in CnAβ1-overexpressing mice. Vessels in Masson's trichrome stainings are indicated by arrows. Bar, 100 μm.

Figure 6. CnAβ1 overexpression enhances infarct vascularisation. A, B, Hearts were isolated 28 days post-infarction and blood vessels were quantified in the infarct region by Masson's trichrome staining (**A**) and immunohistochemistry using anti-αSMA (**B**). **C-E**, CD31 mRNA expression was analysed in the infarct region (**C**), border zone (**D**) and remote area (**E**) by qRT-PCR. **F**, The number of capillaries per field was quantified in the border zone and remote areas of rtTA-CnAβ1 mice stimulated or not with Dox. The graph shows the average number of capillaries in 2 different 40x fields. For all experiments, n=6-15 per group. *p<0.05, **p<0.005, ***p<0.0005 Dox-treated vs no Dox, 1-way ANOVA followed by Dunnett's post-test.

Figure 7. CnAβ1 promotes angiogenesis by inducing VEGF expression through Akt activation. A, Phosphorylation and/or expression of VEGF, HIF1α, CD31, Akt, mTOR and CnAβ1 was analysed by western blot in the remote myocardium. **B-E,** The paracrine angiogenic effect of CnAβ1 was confirmed in angiotube assays. **B,** Neonatal cardiomyocytes from rtTA-CnAβ1 or rtTA mice were treated or not with doxycycline for 72 h and conditioned medium was used to stimulate HUVECs angiotube formation on matrigel. **C,** HUVEC angiotube formation was carried out as in (B) in the presence of a neutralising anti-VEGF or a control antibody. **D,** Angiotube formation was assayed in HUVECs stimulated with conditioned medium from rtTA-CnAβ1 neonatal cardiomyocytes that had been treated with Dox and/or 2 μM rapamycin or DMSO (1:10,000) as a control. B-D, *p<0.05, **p<0.005 +Dox vs –Dox; ##p<0.005 anti-VEGF (C) or Rapamycin (D) vs Control Ab or DMSO, respectively; 2-way ANOVA plus Bonferroni post-test (data in B were analysed with student's t-test). n=3 independent experiments. **E,** VEGF in supernatants in (D) was analysed by western blot.

TABLE

	rtTA-CnAβ1			**rtTA**	
No MI	**No Dox**	**Dox pre-MI**	**Dox post-MI**	**No Dox**	**Dox pre-MI**
EF (%)	66.44±2.41	63.54±2.86	64.24±5.45	63.96±1.58	64.50±2.98
LVESV (μl)	19.30±1.93	24.80±2.75	23.77±5.44	21.69±2.29	23.99±4.03
LVEDV (μl)	58.15±3.69	57.82±3.99	62.90±5.40	56.37±4.56	66.26±7.39
LVPWd (mm)	0.65±0.03	0.67±0.04	0.50±0.04	0.73±0.05	0.71±0.07
IVSs (mm)	0.53±0.03	0.67±0.07	0.51±0.08	0.71±0.06	0.71±0.09
n	14	13	7	8	6

3 days post-MI	No Dox	Dox pre-MI	Dox post-MI	No Dox	Dox pre-MI
EF (%)	40.48±3.37*	38.14±2.70*	29.05±3.86*	35.30±3.06*	31.31±2.98*
LVESV (μl)	35.43±3.82	40.60±5.11	46.68±6.23	28.08±4.10	37.53±2.52
LVEDV (μl)	58.99±4.19	64.63±6.73	64.19±5.79	46.32±4.43	53.52±3.00
LVPWd (mm)	0.77±0.05	0.71±0.08	0.73±0.09	0.71±0.07	0.67±0.05
IVSd (mm)	0.69±0.05	0.66±0.05	0.66±0.05	0.78±0.03	0.71±0.04
n	10	10	7	6	15
28 days post-MI	**No Dox**	**Dox pre-MI**	**Dox post-MI**	**No Dox**	**Dox pre-MI**
EF (%)	37.53±3.62*	56.05±4.99†,‡	55.59±3.06†,‡	33.83±5.38*	36.42±3.06*
LVESV (μl)	58.62±11.00*,‡	32.34±5.05†	28.01±3.57†	48.25±5.57	66.32±11.66*,‡
LVEDV (μl)	90.17±12.93*,‡	72.16±4.70	61.83±4.33†	111.69±34.10*,‡	97.34±12.41‡
LVPWd (mm)	0.82±0.08	0.67±0.05	0.70±0.05	0.72±0.08	0.72±0.05
IVSd (mm)	0.64±0.05	0.51±0.07	0.37±0.04†,‡	0.68±0.06	0.71±0.04
HW/BW (x1000)	5.01±0.23	5.11±0.27	4.50±0.23	5.61±0.49	5.00±0.23
n	10	10	7	6	15

Table 1. CnAβ1 reduces remodelling and improves cardiac function after myocardial infarction and reperfusion. Ligation of the left coronary artery (30' ischemia followed by reperfusion) was performed in rtTA and rtTA-CnAβ1 male transgenic mice and echocardiography analysis was carried out 3 and 28 days later. Mean values ±SE are shown. *p<0.05 infarcted vs no MI, †p<0.05 Dox-treated vs no Dox, 2-way ANOVA plus Bonferroni post-test. ‡p<0.05 28 days post-MI vs 3 days post-MI repeated-measures 2-way ANOVA plus Bonferroni post-test. EF, ejection fraction; LVESV, left ventricular end systolic volume; LVEDV, left ventricular end diastolic volume; LVPWd, left ventricular posterior wall in diastole; IVSd, interventricular septum in diastole; HW/BW, heart weight / body weight ratio; n, number of mice analysed.

Full title: The calcineurin variant CnAβ1 improves post-infarction ventricular remodelling by promoting infarct vascularisation

Running title: CnAβ1 promotes infarct vascularisation

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ABSTRACT

Aims ~~– Myocardial infarction remains a major cause of death and disability worldwide. Dead cardiomyocytes are substituted by a collagen scar that undergoes progressive expansion, leading to ventricular remodelling, loss of contractile capacity and eventually to heart failure. Therapies aimed directly at improving remodelling are therefore necessary. In this work we investigated the effect of the calcineurin splicing variant CnAβ1 on the response to ischemia/reperfusion and heart remodelling.~~ Ventricular remodelling following myocardial infarction progressively leads to loss of contractile capacity and heart failure. Although calcineurin promotes maladaptive cardiac hypertrophy, we recently showed that the calcineurin splicing variant CnAβ1 has beneficial effects on the infarcted heart. However, whether this variant limits necrosis or improves remodelling is still unknown, precluding translation to the clinical arena. Here we explored the effects and therapeutic potential of CnAβ1 overexpression post-infarction. *Methods and Results* - Double transgenic mice with inducible cardiomyocyte-specific overexpression of CnAβ1 underwent left coronary artery ligation followed by reperfusion. Echocardiographic analysis showed depressed cardiac function in all infarcted mice 3 days post-infarction. Induction of CnAβ1 overexpression one week after infarction improved function and reduced ventricular dilatation. CnAβ1-overexpressing mice showed shorter, thicker scars and reduced infarct expansion, accompanied by reduced myocardial remodelling. CnAβ1 induced vascular endothelial growth factor (VEGF) expression in cardiomyocytes, which resulted in increased infarct vascularisation. This paracrine angiogenic effect of CnAβ1 was mediated by activation of the Akt/mTOR pathway and VEGF. *Conclusions* - Our results indicate that CnAβ1 exerts

beneficial effects on the infarcted heart by promoting infarct vascularisation and preventing infarct expansion. These findings highlight the translational potential of CnAβ1 for gene-based therapies.

Keywords: CnAβ1, calcineurin, myocardial infarction, cardiac remodelling, Akt

Myocardial infarction remains a major cause of mortality and hospitalisation worldwide. Reperfusion of the blocked artery through percutaneous coronary intervention or thrombolytic therapy reduces infarct size and increases survival[1]. However, even if reperfusion therapies have allowed a dramatic reduction in immediate mortality, infarction survivors are at high risk of suffering from heart failure, arrhythmia and sudden death. Shortly after reperfusion millions of cardiomyocytes die due to oxidative stress and are substituted by scar tissue. The mechanical stress experienced by the ventricle progressively causes scar thinning and expansion, leading to dilatation of the left ventricle and other structural changes collectively known as remodelling[2, 3]. The surviving cardiomyocytes undergo hypertrophy to compensate the loss of contractile capacity, but these changes progressively lead to a decline in cardiac function and eventually to the development of heart failure.

Far from being a passive tissue, post-infarction scar tissue is populated by different cell types that contribute to maintaining its integrity, regulate extracellular matrix (ECM) turnover and prevent dilatation[2-4]. A few days after infarction a granulation tissue is formed, integrated by leukocytes, new blood vessels, fibroblasts and myofibroblasts that secrete different ECM components, mainly collagen[5]. Inflammatory cells progressively disappear from the area and the scar matures into an ECM-rich fibrotic tissue[6]. The persistence of myofibroblasts in the infarct region contributes to tissue stability. Progressive loss of these cells causes thinning of the infarcted area and left ventricular dilatation.

Whereas a strong ECM in the scar region prevents structural remodelling, collagen accumulation in the remote myocardium has the opposite effect. Interstitial fibrosis increases

passive stiffness, causes electrical remodelling and enhances arrhythmogenicity, further contributing to cardiac remodelling and dysfunction[2]. Therefore, ideal therapies will aim at promoting scar maturation while reducing interstitial fibrosis in the remote myocardium.

The molecular mechanisms involved in post-infarction remodelling are not entirely understood[7]. Calcineurin generally plays a detrimental role in the heart and both protective and deleterious effects have been described for this protein in the response to ischemia/reperfusion[8-10]. It is both sufficient and necessary to induce maladaptive cardiac hypertrophy[11, 12], and activation of the transcription factor NFAT by calcineurin promotes myocardial remodelling and chamber dilatation following infarction[13]. We recently described that, in contrast to other calcineurin A isoforms, the naturally-occurring splicing variant CnAβ1 has a beneficial effect on the heart. CnAβ1 has a unique C-terminal domain, not present in any other known protein, that confers it distinct properties[14]. In a chronic myocardial infarction model with permanent occlusion of the left coronary artery, cardiac-specific overexpression of CnAβ1 has a protective effect, improving cardiac function and reducing long-term scar size[15].

The purpose of the present work was to investigate the potential benefit of CnAβ1 overexpression in response to ischemia/reperfusion injury, to determine whether CnAβ1 is cardioprotective or whether its beneficial action results from post-infarction effects (i.e. improving post-infarction remodelling), and to explore the potential of CnAβ1 for gene-based therapies.

METHODS

Transgenic Mice

rtTA-CnAβ1 mice express the reverse Tet transactivator (rtTA) in a cardiomyocyte-specific manner[16], which in turn induces CnAβ1 overexpression from a second transgene in a doxycycline-inducible fashion (Fig. 1). rtTA mice also express rtTA in a cardiomyocyte-specific manner but lack the CnAβ1 transgene and therefore don't overexpress CnAβ1. The rtTA-CnAβ1 mouse line was generated by crossing the original TetO-CnAβ1 transgenic line, carrying the Tet operator and the CnAβ1 cDNA (Fig. 1) with the rtTA mouse line. Both rtTA-CnAβ1 and rtTA mice were generated in a CBA/Black10 genetic background[16] and inbred in this background for at least eight generations. Male littermates between 3-5 months of age were used for experiments. Doxycycline (Dox) was administered to the mice with the diet (0.3%) starting either 3 weeks before infarction or 1 week after infarction, and maintained until mice were sacrificed.

Surgeries and Echocardiographic analysis

Myocardial infarction was induced in rtTA-CnAβ1 (n=28) and rtTA mice (n=21) by ligation of the left coronary artery for 30 min followed by reperfusion of the artery. Surgeries were carried out under mechanical ventilation with 3-3.5% sevoflurane. Mice received analgesic treatment with buprenorphine (0.3 mg/kg s.c.) after surgery. The mortality rate in the first 24 h post-infarction was 38%; no mortality was found afterwards. Cardiac function, chamber dilatation and wall thickness were analysed by transthoracic echocardiography 3 and 28 days after infarction, as well as in uninfarcted mice, using a Vevo 2100 system and a 45 MHz probe (Visualsonics, Toronto, Canada). Measurements were carried out by a blinded operator

with mice placed on a heating pad under light anaesthesia with sevoflurane adjusted to obtain a target heart rate of 500 ±50 beats per minute[15]. Two-dimensional (2D) and M-mode echocardiography images were recorded in long and short view at the level of the papillary muscles. Left ventricle (LV) end-systolic, end-diastolic volumes and LV ejection fraction were measured from 2D parasternal long axis using the area-length method. Animals were sacrificed by gradually filling the chamber with carbon dioxide.

For the analysis of infarct size using echocardiography, regional left ventricular function was evaluated in the parasternal long-axis view. The left ventricle wall was subdivided into six segments (basal, mid and apical in the anterior and posterior walls). Each segment was scored by an independent blinded evaluator based of its motion and systolic thickening, according to the guidelines of the American Society of Echocardiography[17] (1, normal or hyperkinetic; 2, hypokinetic; 3, akinetic, negligible thickening; 4, dyskinetic, paradoxical systolic motion; 5, aneurysmal, diastolic deformation). The number of dysfunctional segments was quantified and the total score representing the sum of the score of the six individual segments in each heart was calculated.

All experiments were approved by the local Ethics Committee at the Centro Nacional de Investigaciones Cardiovasculares. The investigation conforms to the principles of Laboratory Animal Care which are formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication 85-23, 1996).

Western Blot

Western blot was carried out as previously described[18] using the following primary antibodies: anti-phospho-Akt-Ser473, anti-Akt, anti-phospho-mTOR-Ser2448, anti-mTOR (Cell Signaling), anti-HIF1α and anti-Periostin (Novus Biochemicals), anti-VEGF, anti-Lox and anti-CD31 (Abcam), anti-fibronectin (Sigma). Anti-CnAβ1 has been previously described[14]. Brightness and contrast were linearly adjusted using Photoshop CS5.

Histology and Immunohistochemistry

Samples were fixed in paraformaldehyde (4% in PBS) for 48 h, washed in PBS, dehydrated and included in paraffin. Five microns-thick sections were stained following Masson's trichrome protocol[14]. Images were quantified using ImageJ (NIH, USA). Scar length was determined in Masson's trichrome-stained sections using the midline method, which best correlates with functional measurements[19]. In this method, the infarct length is measured as the length of the midline of the infarcted wall in which >50% of the wall thickness is composed of scar tissue[19]. Scar length represents the percentage of infarct length with respect to the length of the whole LV circumference. Medium/large blood vessels in the infarct region were visually scored in Masson trichrome-stained sections. These vessels would be >20 μm in diameter and typically surrounded by a smooth muscle layer that makes them easily identifiable. In parallel, vessels were stained by immunohistochemistry using anti-αSMA. In both experiments, the number of vessels was determined in the infarct region only and divided by the infarct area. Vessels were quantified in three non-consecutive sections for each mouse. Capillaries were stained using biotin-conjugated Isolectin B4 (Sigma) and scored in two separate 40x microscope fields. Apoptosis in cardiomyocytes (in the whole section) and fibroblasts (in the infarct region) was analysed using Tunel and immunostaining with anti-troponin I (cardiomyocytes) or anti-periostin (fibroblasts).

RNA isolation and qRT-PCR

After sacrificing the animals, mice were perfused with PBS, hearts were excised and samples from the infarct region, border zone and remote myocardium were separated and snap-frozen in liquid nitrogen. Total RNA was isolated using the RNeasy kit from Qiagen, with DNAse digestion on the column. cDNA was synthesized from 100 ng of total RNA using random hexamers and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a 10 μl reaction. Quantitative reverse-transcribed polymerase chain reaction (qRT-PCR) was carried out in an AB9700 thermocycler (Applied Biosystems) using Taqman chemistry or SYBR green (Applied Biosystems). The following Taqman probes were used: Acta1 (Mm00808218_g1), Nppb/BNP (Mm01255770_g1), Col1a1 (Mm00801666_g1), Lox (Mm00495386_m1) Thy1 (Mm00493681_m1), and Acta2 (Mm01204962_gh). Gene expression was normalised to 18S rRNA levels quantified simultaneously using a VIC-labelled probe (4310893E, Applied Biosystems). CnAβ1 and CD31 expression were quantified using SYBR green and the following primers and conditions: CnAβ1 forward: 5'-AGAAGGTGAAGACCAGT-3', CnAβ1 reverse: 5'-AGCAAGTTGCATAACATCATT-3', CD31 forward: AATGGCAACTGGAGCGAGCACT, CD31 reverse: GGAGAAGGCGAGGAGGGTTAGGT; 2 min at 50° C, 10 min at 95° C and 40 cycles of 15 sec at 95° C, 30 sec at 60°. CnAβ2 primers were previously described[15]. qRT-PCR data was analysed using LinReg software in order to estimate the efficiency rates and the Ct values[20].

Angiotubes

Neonatal cardiomyocytes were isolated from rtTA-CnAβ1 and rtTA mice as described[21]. Neonatal mice were sacrificed by cervical dislocation. A total of 750.000 cells/well were seeded in 12-well plates and grown in the presence of 10% fetal calf serum (FCS) for 2 days. Cells were then washed and cultured in serum-free medium for 2 days in the presence of 2 μg/ml doxycycline, 0.1 mM rapamycin or DMSO (1:1000) as indicated in the figures. Conditioned medium was cleared by centrifugation and used for angiotube assays as follows. Matrigel (Becton Dickinson) diluted 1:3 in DMEM was added to 96-well plates (100 μl/well) and allowed to polymerise for 60 min. Human umbilical cord vascular endothelial cells (HUVEC, Promocell)[22] were seeded at a density of 30.000 cells/well in the presence of the different conditioned mediums and allowed to form tubes for 6 h. Where indicated, neutralising anti-VEGF antibody (R&D Systems) was added to the culture (2 ng/μl). Anti-troponin I (Abcam) was used as a negative control antibody. Pictures were taken with a Nikon eclipse TI microscope and the number of tube network nodes was quantified for each well. Experiments were carried out in triplicate and all experiments were repeated at least three times.

Statistics

Data are presented as mean ±SE. In echocardiographic data, the same mice were analysed 3 days and 28 days post-infarction, with non-infarcted animals representing a different group of mice. To test for statistical significance, data were analysed by 2-way ANOVA followed by Bonferroni post-test for multiple comparisons. In addition, a 2-way ANOVA with repeated measures followed by Bonferroni post-test was applied to compare mice at 3 days vs. 28 days post-infarction. Group differences in qRT-PCR and histological quantifications were analysed by 1-way ANOVA followed by Dunnett's post-test to compare to Dox-untreated

mice. Angiotubes were analysed by student's t-test or 2-way ANOVA followed by Bonferroni's post-test depending on the number of variables. Data were analysed with GraphPad Prism 5.0 (Graphpad Software Inc., www.graphpad.com) and differences were considered statistically significant at $p<0.05$.

RESULTS

CnAβ1 improves cardiac function and remodelling after ischemia/reperfusion

To determine the potential benefit of CnAβ1 overexpression in the context of myocardial infarction with reperfusion, we developed double transgenic mice in which CnAβ1 expression is induced 3-4-fold specifically in cardiomyocytes upon administration of Dox in the diet (rtTA-CnAβ1 mice, Fig. ~~S~~1). As negative controls, to test the effect of Dox itself, we used mice that overexpress the same rtTA transactivator but lack the CnAβ1 transgene (rtTA mice). As a first approach we administered Dox starting 3 weeks before surgery and maintained it throughout the experiment. We induced myocardial infarction by occluding the left coronary artery for 30 min. followed by reperfusion, and analysed the mice 3 and 28 days later by echocardiography. rtTA-CnAβ1 and rtTA mice showed functional decline and chamber dilatation 3 days post-infarction regardless of Dox treatment (Fig. ~~1A~~2A-D, Table 1), suggesting that initial infarct size and myocardial loss was analogous among all groups. At day 28 post-infarction we detected a significant improvement in cardiac function (left ventricular ejection fraction [LVEF]) in mice overexpressing CnAβ1 from 3 weeks before surgery (Dox Pre-MI, Fig. ~~1A~~2A, grey bars), while animals not receiving Dox showed no improvement (No Dox, Fig. ~~1A~~2A, white bars). Improved function was accompanied by reduced ventricular dilation in Dox-treated rtTA-CnAβ1 mice (Fig. ~~1C~~2C, Table 1). In contrast, Dox administration to rtTA mice had no effect on cardiac function or chamber dilation (Fig. 2B, 2D). Of note, neither CnAβ1 nor CnAβ2 (the CnAβ splicing isoform that carries the full CnA autoinhibitory domain) mRNA expression showed much variation in response to myocardial infarction itself (Fig. ~~S~~1G).

CnAβ1 induces recovery, rather than protection

The results obtained at 3 days post-infarction suggested that CnAβ1 offered no protection against reperfusion injury whereas the improvement observed at 28 days suggested a positive effect post-infarction. To further discriminate between the protective and recovery effects of CnAβ1 overexpression, we treated rtTA-CnAβ1 mice with Dox starting one week post-infarction, once the scar has developed (Dox post-MI, black bars). Interestingly, a significant enhancement of cardiac function was observed in these mice (Fig ~~1A~~2A), which was accompanied by reduced ventricular dilatation (Fig. ~~1C~~2C). Importantly, in all the study the same mice were sequentially analysed at 3 and 28 days. The functional decline observed at 3 days post-infarction and the subsequent improvement observed at 28 days in Dox-treated rtTA-CnAβ1 mice (Fig. ~~2~~S1, Table 1), demonstrates that CnAβ1 induces functional recovery, rather protection from infarction.

CnAβ1 prevents infarct expansion and improves remote myocardium remodelling

We next analysed the impact that CnAβ1 overexpression has on infarct expansion. We found that overexpression of CnAβ1 before infarction resulted in reduced scar length 28 days post-infarction, compared to mice untreated with Dox (Fig. 3A, Fig. S2). Importantly, a similar effect was achieved when CnAβ1 was induced after infarction. In Dox-treated rtTA-CnAβ1 mice, scar length at 28 days post-infarction (20.47% ±3.85 in mice treated pre-MI; 14.23% ±4.16 in mice treated post-MI) remained similar to that observed at 7 days in untreated mice (21.52% ±2.90). Echocardiographic analysis showed that CnAβ1 overexpression results in a reduction in the number of left ventricle segments with dysfunctional motility 28 days after

infarction (Table S1). This was particularly evident in the mid anterior segment, confirming the reduced infarct size observed by histological methods. In addition, CnAβ1-overexpressing mice showed thicker scars than control mice (Fig. 3B), suggesting that CnAβ1 prevents infarct expansion. Induction of CnAβ1 also resulted in higher expression of collagen Iα1 in the scar region, together with increased expression of lysil oxydase, which crosslinks collagen and elastin molecules into mature fibres, the fibroblast proliferation marker Thy1/CD90 and α-smooth muscle actin (Fig. 3C-F). We also detected increased expression of periostin, fibronectin and lysil oxidase proteins in the infarct region of CnAβ1 overexpressing mice (Fig. 3G). rtTA mice showed no changes in scar length, thickness or expression of fibrosis markers upon Dox administration (Fig. 3A-G, Table S1). To investigate whether reduced infarct expansion was accompanied by improved fibroblast survival, we quantified the percentage of apoptotic fibroblasts by Tunel staining. As shown in Fig. ~~S4A~~S3A, only a very low degree of fibroblast apoptosis was detected in the infarct region 28 days post-infarction and no significant difference among the groups was observed.

To determine whether changes in infarct expansion were paralleled by improved remodelling of the remote myocardium, we analysed the cardiomyocyte area and the expression of heart failure markers in this region. CnAβ1 overexpression either before or after infarction significantly reduced the cross-sectional area of cardiomyocytes and the wall thickness in the remote myocardium (Fig. 4A, Table 1). This was accompanied by a significant reduction of α-skeletal actin, BNP and collagen Iα1 in the remote myocardium of these mice (Fig. 4B-D). A trend towards reduced cardiomyocyte apoptosis was also detected in CnAβ1-overexpressing mice, although the percentage of apoptotic cardiomyocytes 28 days post-infarction was low (Fig. ~~S4B~~S3B). In contrast, Dox administration to rtTA mice had no effect on cardiomyocyte size or expression, heart failure or fibrosis markers or apoptosis (Fig. 4A-D, Fig. ~~S4B~~S3B).

Improved vascularisation in the infarct region of CnAβ1-overexpressing mice

The effect of CnAβ1 on infarcted hearts is reminiscent of cardiomyocyte activation of the hypoxia inducible factor 1α (HIF1α), which drives expression of angiogenic factors and promotes infarct vascularisation[23]. To determine whether more active, thicker scars were supported by enhanced vascularisation in CnAβ1-overexpressing mice, we quantified the amount of blood vessels in the scar region. We observed that induction of CnAβ1 overexpression either pre- or post-infarction increased the number of blood vessels in the infarcted region (Fig. ~~S3~~5, Fig. ~~5A~~6A, ~~5B~~6B). This was confirmed by an upregulation of CD31 and αSMA mRNA in the same region (Fig. ~~5B~~6C, Fig. 3F, 3G). A mild induction in CD31 mRNA expression and in the number of capillaries was detected also in the remote area upon CnAβ1 induction (Fig. ~~5E~~6E, ~~5F~~6F).

CnAβ1 promotes vascularisation by activating the Akt signalling pathway

We have previously shown that CnAβ1 activates the Akt pathway through its unique C-terminal domain[15]. Akt is known to induce angiogenesis in the heart by promoting expression of VEGF[24]. We therefore investigated the role of this signalling pathway in the angiogenic response elicited by CnAβ1. We observed that Dox stimulation of rtTA-CnAβ1 mice induced expression of VEGF and the hypoxia inducible factor 1α (HIF1α) in the remote myocardium (Fig. ~~6A~~7A). This was accompanied by increased CD31 expression and activation of the Akt/mTOR pathway. No changes were observed in rtTA mice upon Dox treatment.

To test whether the paracrine angiogenic effect of CnAβ1 was reproduced in culture, we used the assay for HUVEC angiotube formation on matrigel[25]. As shown in Fig. ~~6B~~7B,

conditioned medium from Dox-treated rtTA-CnAβ1 cardiomyocytes stimulated angiotube formation by HUVEC, whereas that of untreated cells or rtTA cardiomyocytes had no effect. This effect was blocked by a neutralising anti-VEGF antibody (Fig. ~~6C~~7C), suggesting that VEGF mediates the paracrine effect of CnAβ1-overexpressing cardiomyocytes on endothelial cells. Interestingly, treatment of rtTA-CnAβ1 cardiomyocytes with the inhibitor of the Akt/mTOR pathway rapamycin blocked the induction of angiotube formation by the conditioned medium (Fig. ~~6D~~7D). Rapamycin also prevented the induction of VEGF secretion by Dox-treated rtTA-CnAβ1 cardiomyocytes (Fig. ~~6E~~7E). These results suggest that CnAβ1 activates Akt/mTOR in cardiomyocytes to induce VEGF secretion and paracrine activation of angiogenesis.

DISCUSSION

We show here that, in contrast to other calcineurin isoforms, CnAβ1 has beneficial effects on the heart after reperfusion injury. CnAβ1 reduces ventricular dilatation and improves function even when overexpressed one week after infarction. By activating the Akt/mTOR pathway, CnAβ1 promotes secretion of angiogenic mediators by cardiomyocytes and enhances vascularisation of the infarct region, thus precluding infarct expansion and improving heart remodelling.

Our results suggest that the beneficial effects of CnAβ1 overexpression involve the prevention of heart remodelling, rather than cardioprotection. The significant ventricular dilatation and decline in cardiac function observed here at day 3 post-infarction in all experimental groups strongly suggests that CnAβ1 exerts its beneficial effects by improving myocardial remodelling after infarction, rather than by protecting from reperfusion injury. This is further supported by the fact that induction of CnAβ1 as late as 1 week post-infarction improves cardiac function and reduces ventricular dilatation. The scar tissue in CnAβ1-overexpressing mice is thicker and richer in collagen, matrix crosslinking enzymes and activated fibroblasts, which results in reduced infarct expansion.

We previously showed that CnAβ1 overexpression leads to reduced scar formation after permanent occlusion of the left coronary artery[15]. In the chronic infarction model, scars from CnAβ1-overexpressing mice showed reduced fibroblast number and collagen expression. In contrast, Dox-treated rtTA-CnAβ1 show thicker scars with increased collagen expression after ischemia reperfusion. This apparent discrepancy is likely due to the different response to permanent ischemia and to reperfusion injury. Chronic myocardial infarction

eventually results in the death of most cells in the infarct region, leading to loss of extracellular matrix production and infarct expansion. In that context, the reduced infarct size observed in the presence of CnAβ1 is likely due to reduced myocardial damage, which is consistent with the improved function already observed 7 days post-infarction[15]. In contrast, reperfusion injury reduces cardiomyocyte death and promotes a stronger angiogenic response in the infarcted myocardium[26]. Our results suggest that CnAβ1 promotes VEGF expression from cardiomyocytes to improve vascularization of the infarcted region, while stimulating fibroblast proliferation and ECM production in this area. In addition, although we only detected a very low degree of fibroblast apoptosis 28 days post-infarction, we can not exclude the possibility that better vascularization improves fibroblast survival at earlier time points. The increase in fibroblasts and myofibroblasts in the scar tissue will contribute to increased extracellular matrix turnover and to the maintenance of the scar structure, thus preventing remodelling. Distinct effects on heart remodelling in response to permanent ischemia and reperfusion injury have been described for other factors like GDF15[27].

In addition, the remote myocardium of CnAβ1-overexpressing mice shows reduced cardiomyocyte hypertrophy, together with lower expression of collagen and heart failure markers. This reduction in myocardial remodelling is likely the result of the limited infarct expansion. Importantly, all these effects were observed even when CnAβ1 overexpression was induced late after infarction, indicating that CnAβ1's main target is not the reduction of cardiomyocyte death or inflammation, but the prevention of infarct expansion and chamber dilatation.

Molecular therapies capable of improving ventricular function after infarction are scarce. Overexpression of sonic hedgehog starting 5 days post-infarction attenuates remodelling and improves function[28]. Inhibition of Wnt signalling with Frizzled antagonist peptides reduces infarct expansion and prevents ventricular dilatation[29]. This effect was also

achieved when the peptide was administered two weeks after infarction, suggesting that Wnt signalling has a detrimental effect on heart remodelling rather than on early infarct healing. In both cases, the improvement in cardiac remodelling was accompanied by enhanced infarct vascularisation. In this same regard, overexpression of the angiogenesis regulator HIF1α also improves vascularisation and function, and reduces infarct size in transgenic mice[23]. However, chronic overexpression of HIF1α has detrimental effects on the heart[30]. Similarly, short-term Akt activation in cardiomyocytes induces VEGF and angiogenesis but sustained activation induces maladaptive hypertrophy and fibrosis[24]. We show here that CnAβ1 induces angiogenesis by promoting VEGF expression in an Akt/mTOR-dependent manner. In contrast to sustained Akt activation, CnAβ1 overexpression in cardiomyocytes improves infarct vascularisation, cardiac remodelling and function without inducing the detrimental side effects observed after sustained Akt or HIF1α overexpression.

Together our results underline the therapeutic potential of CnAβ1 and suggest that it may be an excellent candidate for gene therapies aimed at reducing ventricular remodelling and improving cardiac function post-infarction. We demonstrate that these beneficial effects can be achieved by inducing CnAβ1 expression late after infarction. Our work constitutes a first step for the translation of these findings to the clinical setting.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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FIGURE LEGENDS

Figure 1. Cardio-specific inducible transgenic mice used in this study. A, rtTA-CnAβ1 mice overexpress the reverse Tet transactivator (rtTA) specifically in cardiomyocytes under the control of the Xenopus MLC2v promoter. Upon doxycycline (Dox) administration in the diet, rtTA activates overexpression of CnAβ1 from a second transgene. **B**, Schematic showing the different experimental groups and Dox administration regime. **C**, CnAβ1 mRNA expression was analysed in the remote myocardium 28 days post-infarction by qRT-PCR. Results are expressed as mean fold induction ±SE over the values of uninjured hearts (indicated by a dashed line). *p<0.05 Dox-treated vs no Dox. **D**, rtTA mice were used as negative controls to test for the effect of rtTA overexpression and Dox administration themselves. These mice overexpress rtTA in cardiomyocytes but not CnAβ1, since they lack the second transgene present in rtTA-CnAβ1 mice. **E**, Schematic showing the rtTA mouse groups and Dox administration regime. **F**, Analysis of CnAβ1 mRNA expression in the remote myocardium 28 days post-infarction by qRT-PCR. Results are expressed as mean fold induction ±SE over the values of uninjured hearts (dashed line). n=6-15 per group. **G**, qRT-PCR analysis of CnAβ1 and CnAβ2 mRNA expression in uninjured hearts and in the remote and infarct regions of the heart 28 days post-infarction. *p<0.05 compared to no infarction, 1-way ANOVA plus Bonferroni post-test.

Figure ~~1~~2. Induction of CnAβ1 overexpression late after ischemia/reperfusion improves cardiac function and reduces dilatation. A Tet-on inducible transgenic mouse with

cardiomyocyte-specific expression of the reverse Tet transactivator (rtTA) was used. CnAβ1 overexpression was induced in rtTA-CnAβ1 mice with doxycycline starting either 3 weeks before (Dox pre-MI) or 1 week after (Dox post-MI) ischemia/reperfusion (**A, C**). Control rtTA mice (**B, D**) lack the CnAβ1 transgene and therefore do not overexpress CnAβ1 upon doxycycline treatment. Ejection fraction (**A, B**) and left ventricular end systolic volume (**C, D**) were analysed by echocardiography 3 and 28 days post-infarction. Graphs show means ±SE. n=6-15 per group. *p<0.05 infarcted vs no MI, ***p<0.0005 infarcted vs no MI, #p<0.05 Dox-treated vs no Dox, 2-way ANOVA plus Bonferroni post-test. §p<0.05 28 days post-MI vs 3 days post-MI repeated-measures 2-way ANOVA followed by Bonferroni post-test.

~~**Figure 2. Echocardiographic analysis shows post-infarction functional improvement after CnAβ1 overexpression in the same animal.** CnAβ1 overexpression was induced in rtTA-CnAβ1 mice with doxycycline starting either 3 weeks before (Dox pre-MI) or 1 week after (Dox post-MI) ischemia/reperfusion. rtTA mice either treated or untreated with doxycycline were used as negative controls. M-Mode echocardiographic analysis in the long axis was carried out 3 and 28 days post-infarction. Images are shown for one mouse per group, the same mouse at both time points. Note that mice from all groups show poor contraction of the anterior wall at 3 days.~~

Figure 3. CnAβ1 reduces infarct expansion. A, B Scar length (A) and thickness (B) were analysed 28 days post-infarction using histological methods. **C-F**, mRNA expression of collagen Iα1 (C), lysyl oxidase (D), Thy1 (E) and α-smooth muscle actin (F) were analysed by qRT-PCR in the infarct region only. Results are expressed as mean fold induction ±SE

over the values of uninjured hearts (dashed line). n=6-15 per group. *p<0.05 Dox-treated vs no Dox, 1-way ANOVA followed by Dunnett's post-test. **G**, Western blot analysis of lysyl oxidase (Lox), fibronectin (Fn), periostin (Postn) and CD31 in the infarct region of rtTA-CnAβ1 and rtTA mice 28 days post-infarction.

Figure 4. Reduced myocardial remodelling in CnAβ1-overexpressing mice. A, Cardiomyocyte cross-sectional area was analysed 28 days post-infarction by histological methods. **B-D**, The expression of α-skeletal actin (B), BNP (C) and collagen Iα1 (D) mRNA was analysed by qRT-PCR in the remote myocardium. Results are expressed as mean fold induction ±SE over the values of uninjured hearts (dashed line). n=6-15 per group. *p<0.05 Dox-treated vs no Dox, 1-way ANOVA followed by Dunnett's post-test.

Figure 5. Reduced infarct expansion in CnAβ1-overexpressing mice is accompanied by improved infarct vascularisation. rtTA-CnAβ1 and rtTA mice, either treated or untreated with doxycycline, were sacrificed 28 days post-infarction. Hearts were excised, fixed and stained with Masson's trichrome protocol (left and centre) or with anti-αSMA and DAPI (right). Histological sections of the infarct region show an increase in the number of medium and large vessels in CnAβ1-overexpressing mice. Vessels in Masson's trichrome stainings are indicated by arrows. Bar, 100 μm.

Figure ~~5~~6. CnAβ1 overexpression enhances infarct vascularisation. A, B, Hearts were isolated 28 days post-infarction and blood vessels were quantified in the infarct region by Masson's trichrome staining (**A**) and immunohistochemistry using anti-αSMA (**B**). **C-E**,

CD31 mRNA expression was analysed in the infarct region (**C**), border zone (**D**) and remote area (**E**) by qRT-PCR. **F**, The number of capillaries per field was quantified in the border zone and remote areas of rtTA-CnAβ1 mice stimulated or not with Dox. The graph shows the average number of capillaries in 2 different 40x fields. For all experiments, n=6-15 per group. *p<0.05, **p<0.005, ***p<0.0005 Dox-treated vs no Dox, 1-way ANOVA followed by Dunnett's post-test.

Figure ~~6~~7. CnAβ1 promotes angiogenesis by inducing VEGF expression through Akt activation. A, Phosphorylation and/or expression of VEGF, HIF1α, CD31, Akt, mTOR and CnAβ1 was analysed by western blot in the remote myocardium. **B-E,** The paracrine angiogenic effect of CnAβ1 was confirmed in angiotube assays. **B,** Neonatal cardiomyocytes from rtTA-CnAβ1 or rtTA mice were treated or not with doxycycline for 72 h and conditioned medium was used to stimulate HUVECs angiotube formation on matrigel. **C,** HUVEC angiotube formation was carried out as in (B) in the presence of a neutralising anti-VEGF or a control antibody. **D,** Angiotube formation was assayed in HUVECs stimulated with conditioned medium from rtTA-CnAβ1 neonatal cardiomyocytes that had been treated with Dox and/or 2 μM rapamycin or DMSO (1:10,000) as a control. B-D, *p<0.05, **p<0.005 +Dox vs –Dox; ##p<0.005 anti-VEGF (C) or Rapamycin (D) vs Control Ab or DMSO, respectively; 2-way ANOVA plus Bonferroni post-test (data in B were analysed with student's t-test). n=3 independent experiments. **E,** VEGF in supernatants in (D) was analysed by western blot.

TABLE

	rtTA-CnAβ1			rtTA	
No MI	**No Dox**	**Dox pre-MI**	**Dox post-MI**	**No Dox**	**Dox pre-MI**
EF (%)	66.44±2.41	63.54±2.86	64.24±5.45	63.96±1.58	64.50±2.98
LVESV (μl)	19.30±1.93	24.80±2.75	23.77±5.44	21.69±2.29	23.99±4.03
LVEDV (μl)	58.15±3.69	57.82±3.99	62.90±5.40	56.37±4.56	66.26±7.39
LVPWd (mm)	0.65±0.03	0.67±0.04	0.50±0.04	0.73±0.05	0.71±0.07
IVSs (mm)	0.53±0.03	0.67±0.07	0.51±0.08	0.71±0.06	0.71±0.09
n	14	13	7	8	6
3 days post-MI	**No Dox**	**Dox pre-MI**	**Dox post-MI**	**No Dox**	**Dox pre-MI**
EF (%)	40.48±3.37*	38.14±2.70*	29.05±3.86*	35.30±3.06*	31.31±2.98*
LVESV (μl)	35.43±3.82	40.60±5.11	46.68±6.23	28.08±4.10	37.53±2.52
LVEDV (μl)	58.99±4.19	64.63±6.73	64.19±5.79	46.32±4.43	53.52±3.00
LVPWd (mm)	0.77±0.05	0.71±0.08	0.73±0.09	0.71±0.07	0.67±0.05
IVSd (mm)	0.69±0.05	0.66±0.05	0.66±0.05	0.78±0.03	0.71±0.04
n	10	10	7	6	15
28 days post-MI	**No Dox**	**Dox pre-MI**	**Dox post-MI**	**No Dox**	**Dox pre-MI**
EF (%)	37.53±3.62*	56.05±4.99†,‡	55.59±3.06†,‡	33.83±5.38*	36.42±3.06*
LVESV (μl)	58.62±11.00*,‡	32.34±5.05†	28.01±3.57†	48.25±5.57	66.32±11.66*,‡
LVEDV (μl)	90.17±12.93*,‡	72.16±4.70	61.83±4.33†	111.69±34.10*,‡	97.34±12.41‡
LVPWd (mm)	0.82±0.08	0.67±0.05	0.70±0.05	0.72±0.08	0.72±0.05
IVSd (mm)	0.64±0.05	0.51±0.07	0.37±0.04†,‡	0.68±0.06	0.71±0.04
HW/BW (x1000)	5.01±0.23	5.11±0.27	4.50±0.23	5.61±0.49	5.00±0.23
n	10	10	7	6	15

Table 1. CnAβ1 reduces remodelling and improves cardiac function after myocardial infarction and reperfusion. Ligation of the left coronary artery (30' ischemia followed by reperfusion) was performed in rtTA and rtTA-CnAβ1 male transgenic mice and echocardiography analysis was carried out 3 and 28 days later. Mean values ±SE are shown. *p<0.05 infarcted vs no MI, †p<0.05 Dox-treated vs no Dox, 2-way ANOVA plus Bonferroni post-test. ‡p<0.05 28 days post-MI vs 3 days post-MI repeated-measures 2-way ANOVA plus Bonferroni post-test. EF, ejection fraction; LVESV, left ventricular end systolic volume; LVEDV, left ventricular end

diastolic volume; LVPWd, left ventricular posterior wall in diastole; IVSd, interventricular septum in diastole; HW/BW, heart weight / body weight ratio; n, number of mice analysed.

Figure 1
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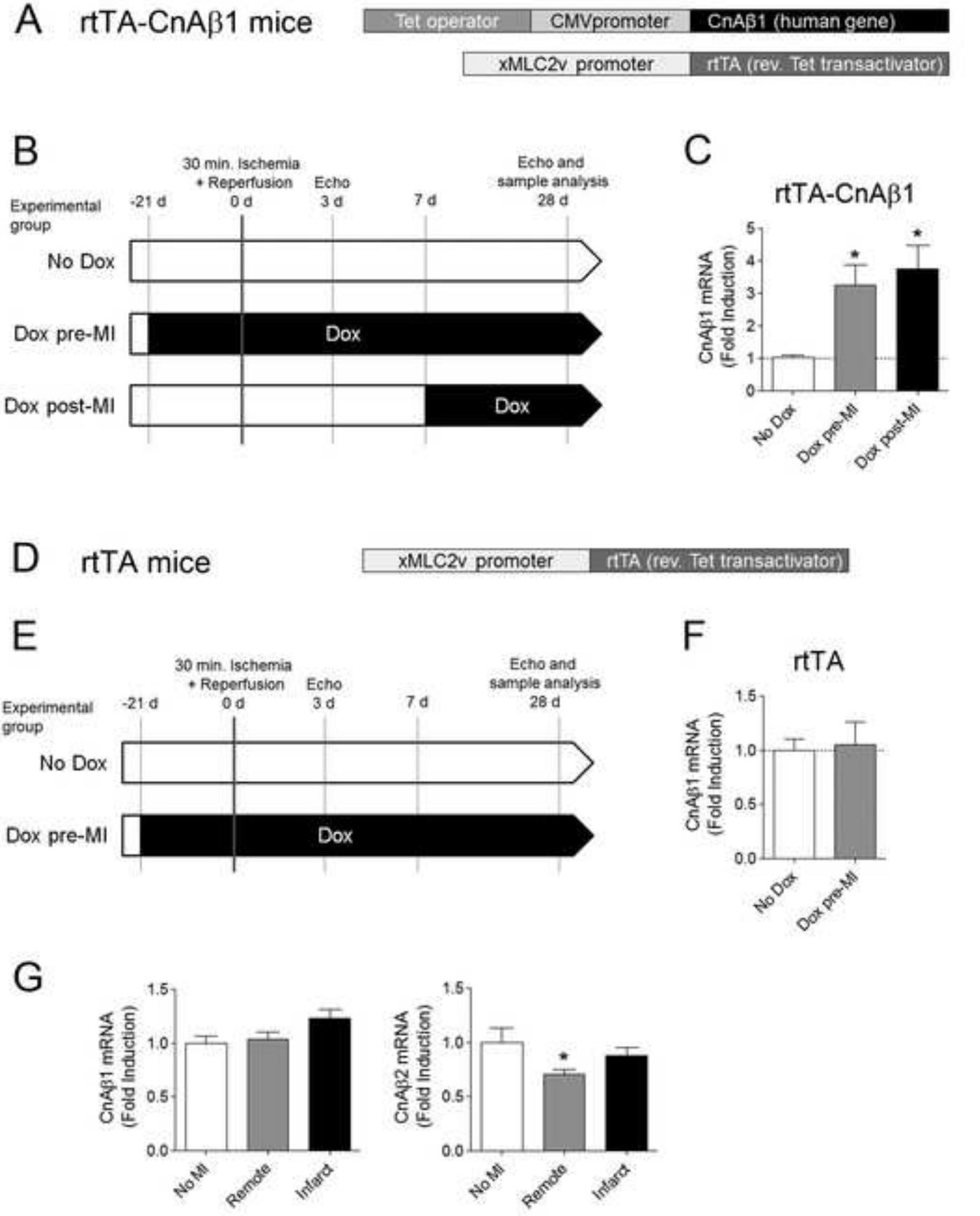

López-Olañeta, Fig. 1.

Figure 2
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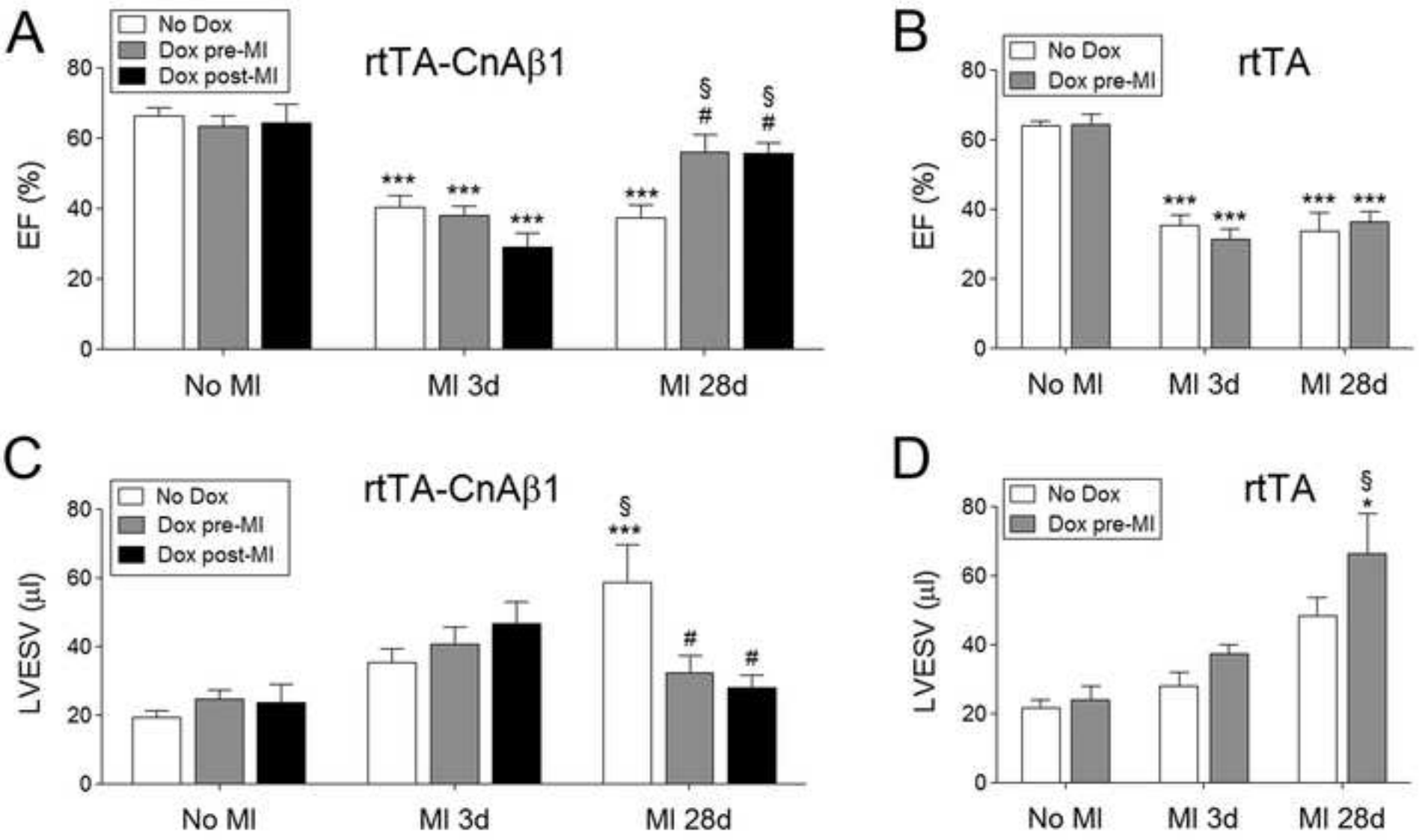

López-Olañeta et al., Fig. 2

Figure 3
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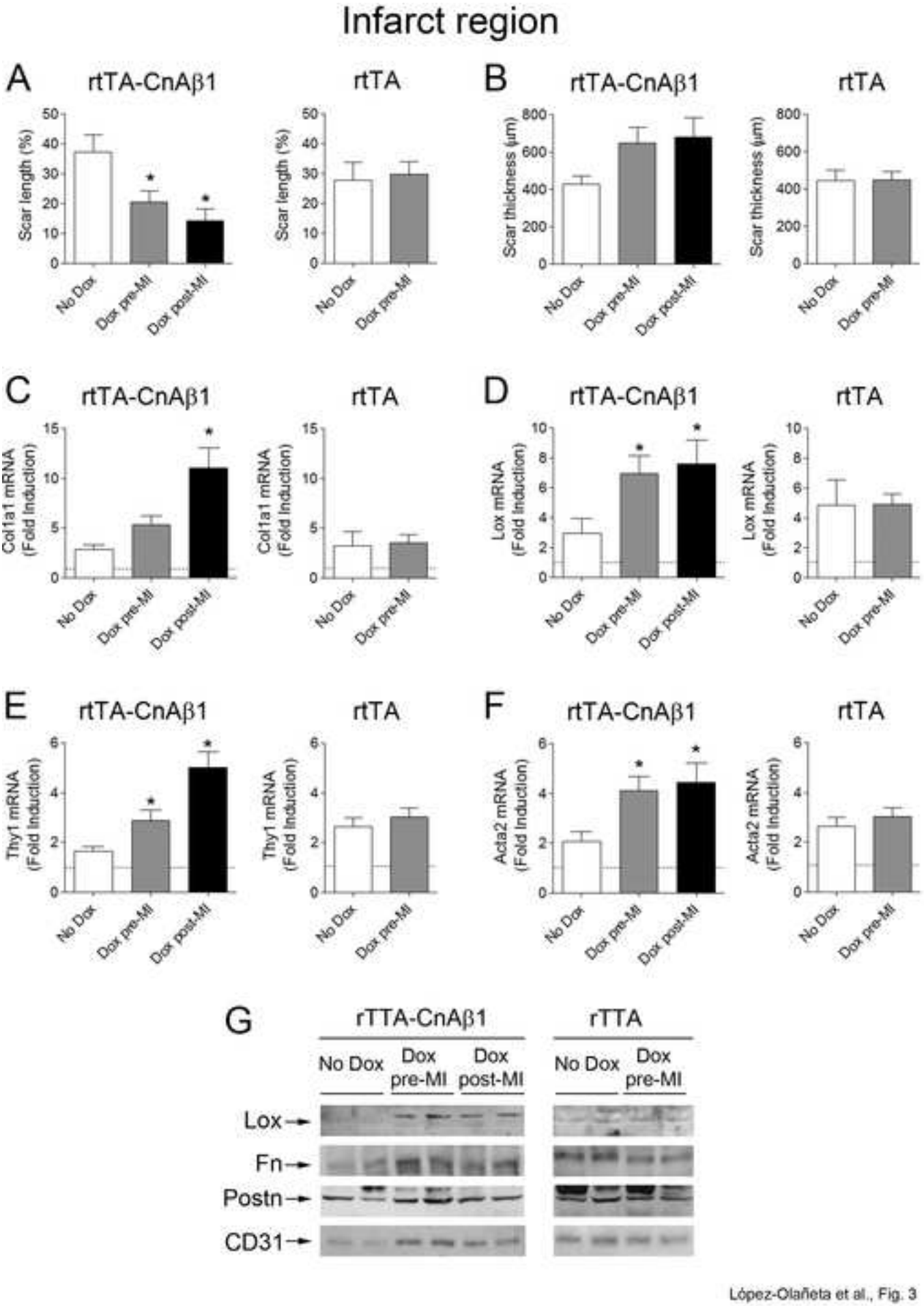

López-Olañeta et al., Fig. 3

Figure 4
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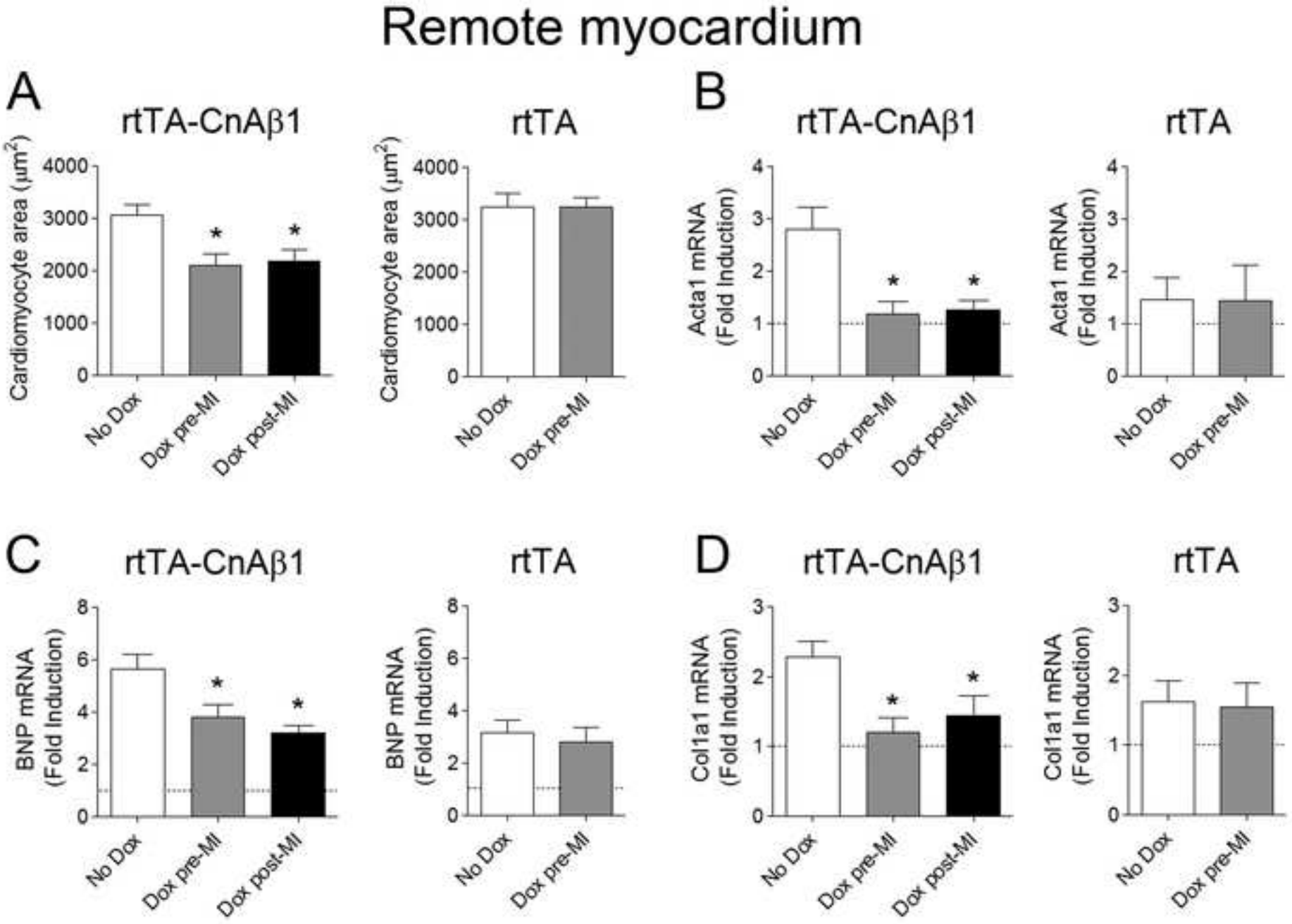

López-Olañeta et al., Fig. 4

Figure 5
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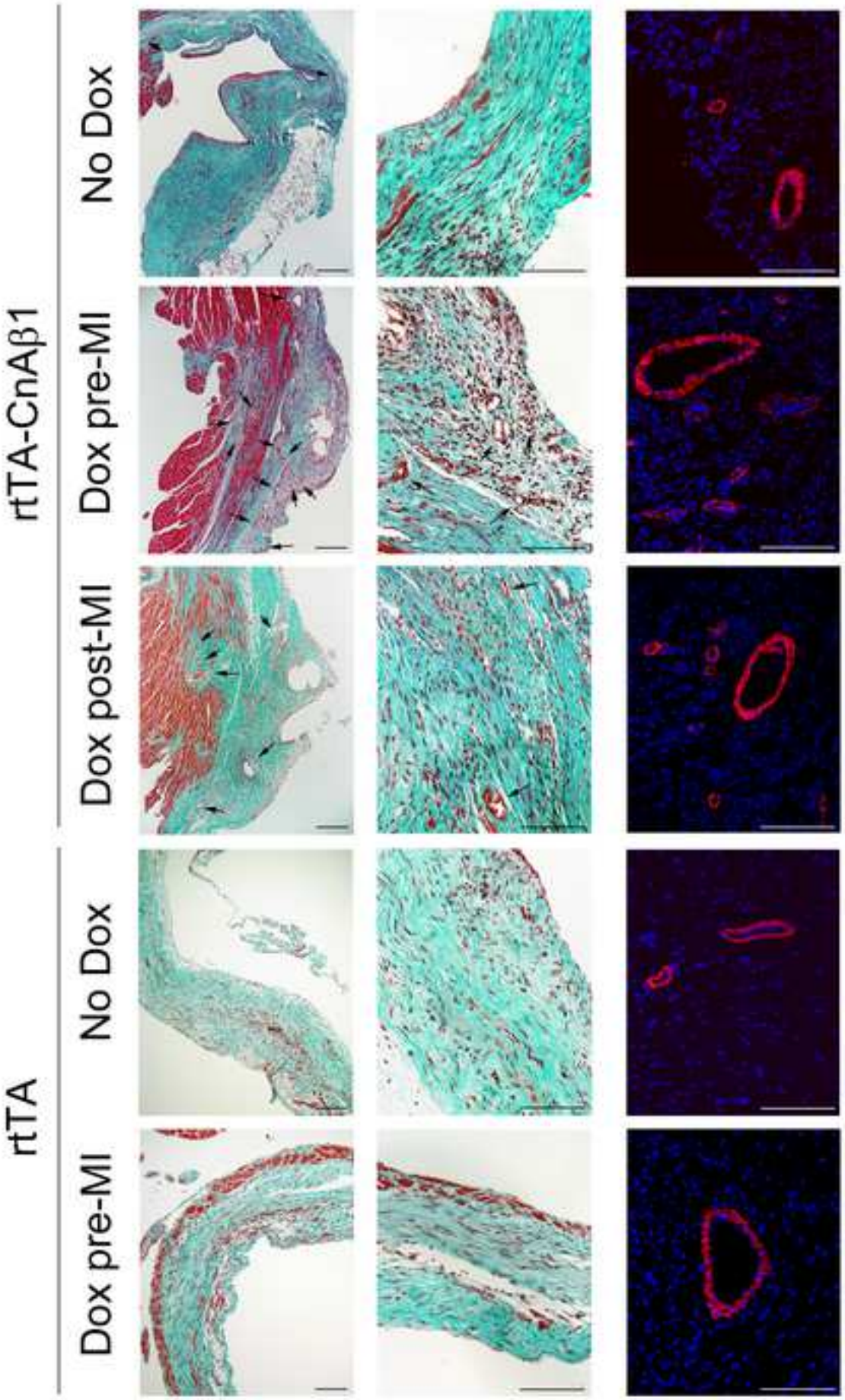

López-Olañeta et al., Fig. 5

Figure 6
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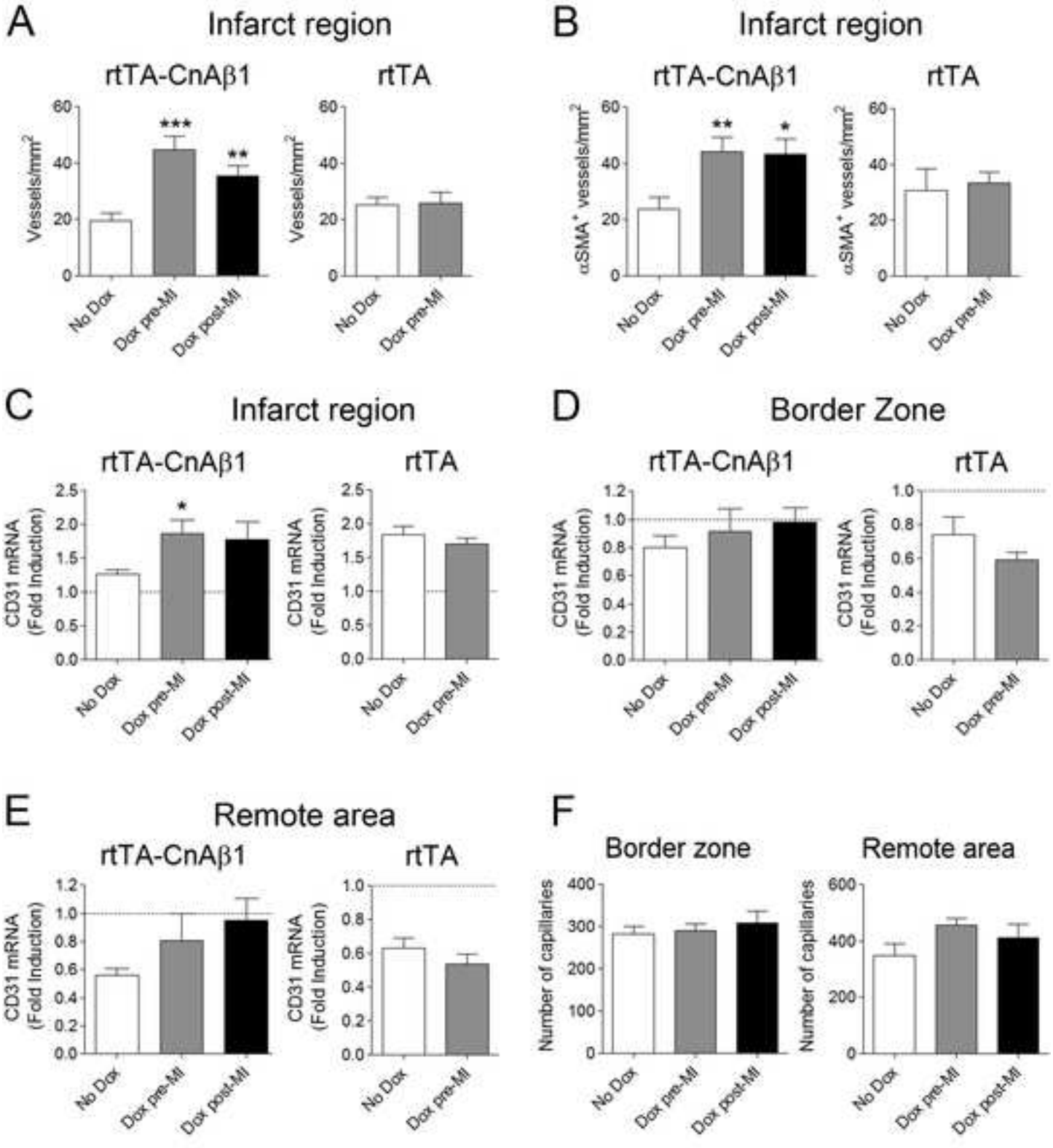

López-Olañeta et al., Fig. 6

Figure 7
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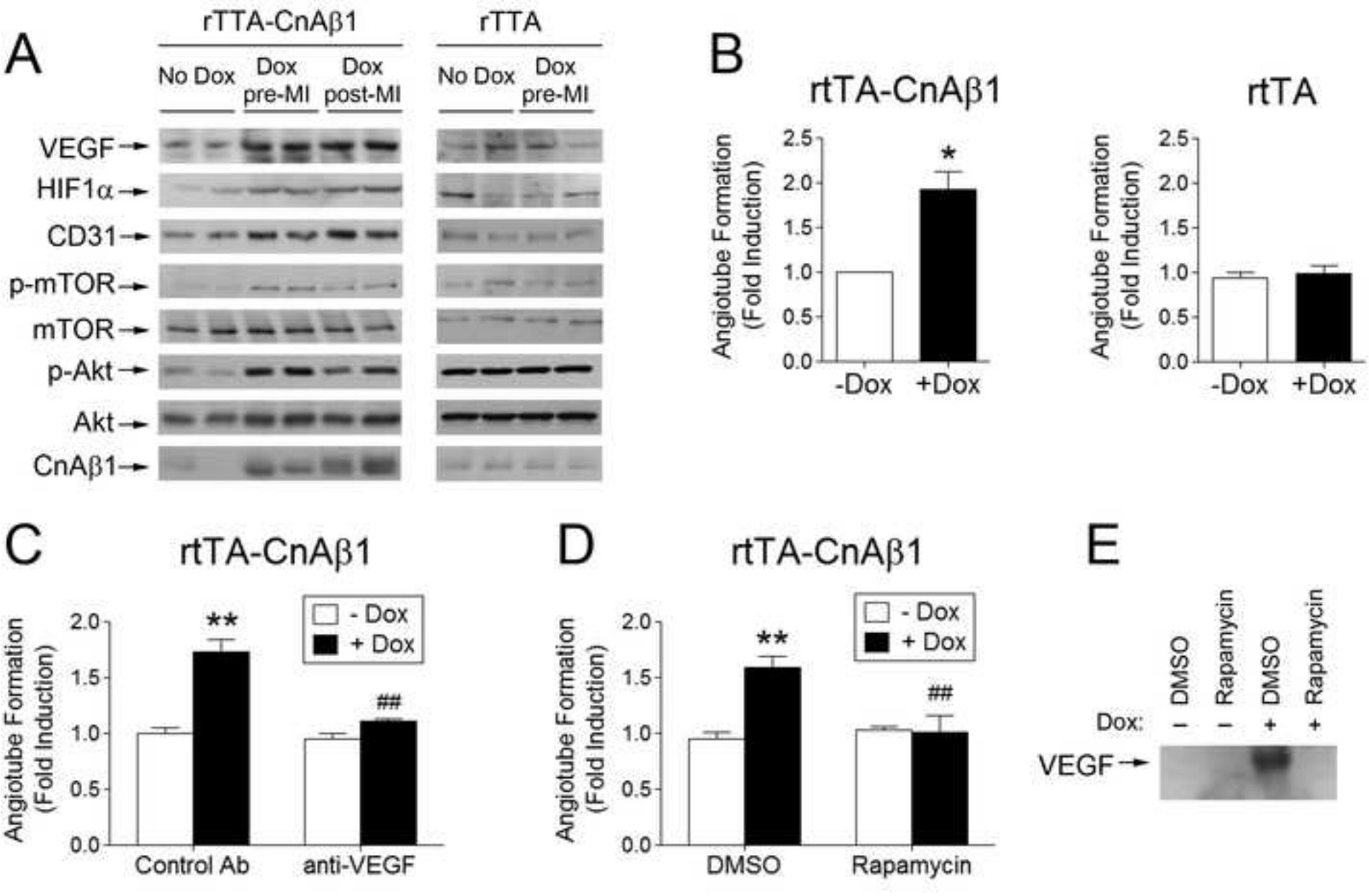


López-Olañeta et al., Fig. 7

SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE

TABLE

	rtTA-CnAβ1			rtTA	
LV segment	**No Dox**	**Dox pre-MI**	**Dox post-MI**	**No Dox**	**Dox pre-MI**
Anterior basal	1.2±0.20	1.0±0	1.1±0.14	1.0±0	1.0±0
Anterior mid	2.5±0.34	1.0±0*	1.1±0.14*	2.0±0.45	2.3±0.32
Anterior apical	3.3±0.42	2.3±0.42	2.4±0.72	3.0±0.89	2.5±0.42
Posterior basal	1.1±0.1	1.0±0	1.0±0	1.2±0.2	1.1±0.07
Posterior mid	1.9±0.28	1.3±0.15	1.3±0.18	2.2±0.49	1.9±0.25
Posterior apical	2.8±0.33	2.1±0.43	1.7±0.36	2.4±0.60	2.2±0.31
n Dysfunctional segments	3.3±0.45	1.4±0.43*	1.43±0.75*	2.6±0.93	2.4±0.40
Total score	12.8±1.03	8.7±0.91*	8.7±1.32*	11.2±1.80	11.0±1.09
n	10	10	7	6	15

Table S1. CnAβ1 improves cardiac motility and reduces infarct size after myocardial infarction and reperfusion. Ligation of the left coronary artery (30' ischemia followed by reperfusion) was performed in rtTA and rtTA-CnAβ1 transgenic mice and echocardiography analysis was carried out 28 days later in the long axis. Regional left ventricular function was evaluated by echocardiography in the parasternal long-axis view and scored as follows: 1, normal or hyperkinesis; 2, hypokinesis; 3, akinesis (negligible thickening); 4, dyskinesis (paradoxical systolic motion); 5, aneurysmal (diastolic deformation). Mean values ±SE are shown for each of the six LV segments. n Dysfunctional segments represents the number of segments with abnormal function. Total score represents the sum of the score of the six segments. *$p<0.05$ Dox-treated vs no Dox (student's t-test for rtTA mice and 1-way ANOVA followed by Bonferroni post-test for rtTA-CnAβ1 mice).

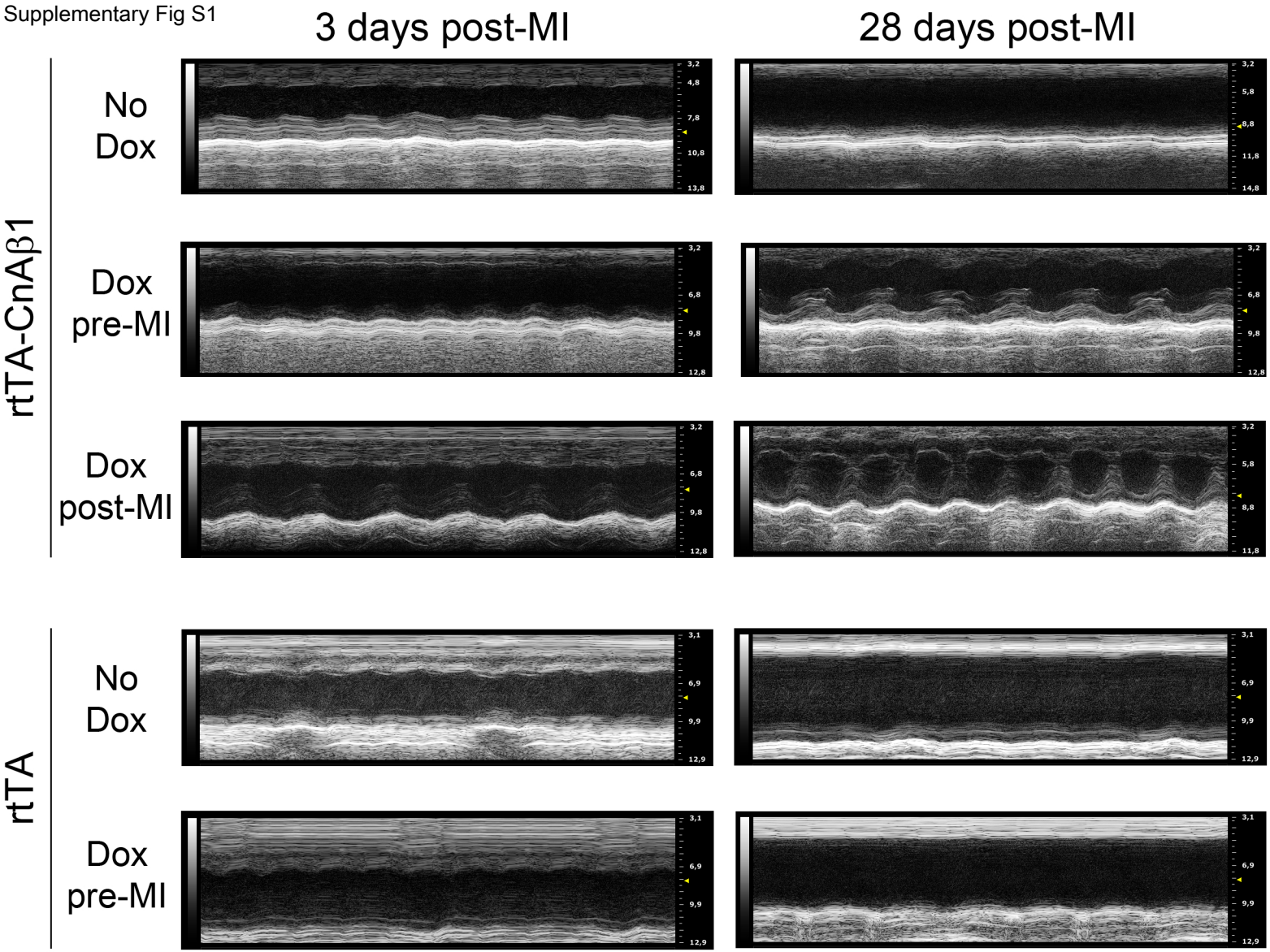
SUPPLEMENTARY FIGURES

Figure S1. Echocardiographic analysis shows post-infarction functional improvement after CnAβ1 overexpression in the same animal. CnAβ1 overexpression was induced in rtTA-CnAβ1 mice with doxycycline starting either 3 weeks before (Dox pre-MI) or 1 week after (Dox post-MI) ischemia/reperfusion. rtTA mice either treated or untreated with doxycycline were used as negative controls. M-Mode echocardiographic analysis in the long axis was carried out 3 and 28 days post-infarction. Images are shown for one mouse per group, the same mouse at both time points. Note that mice from all groups show poor contraction of the anterior wall at 3 days.

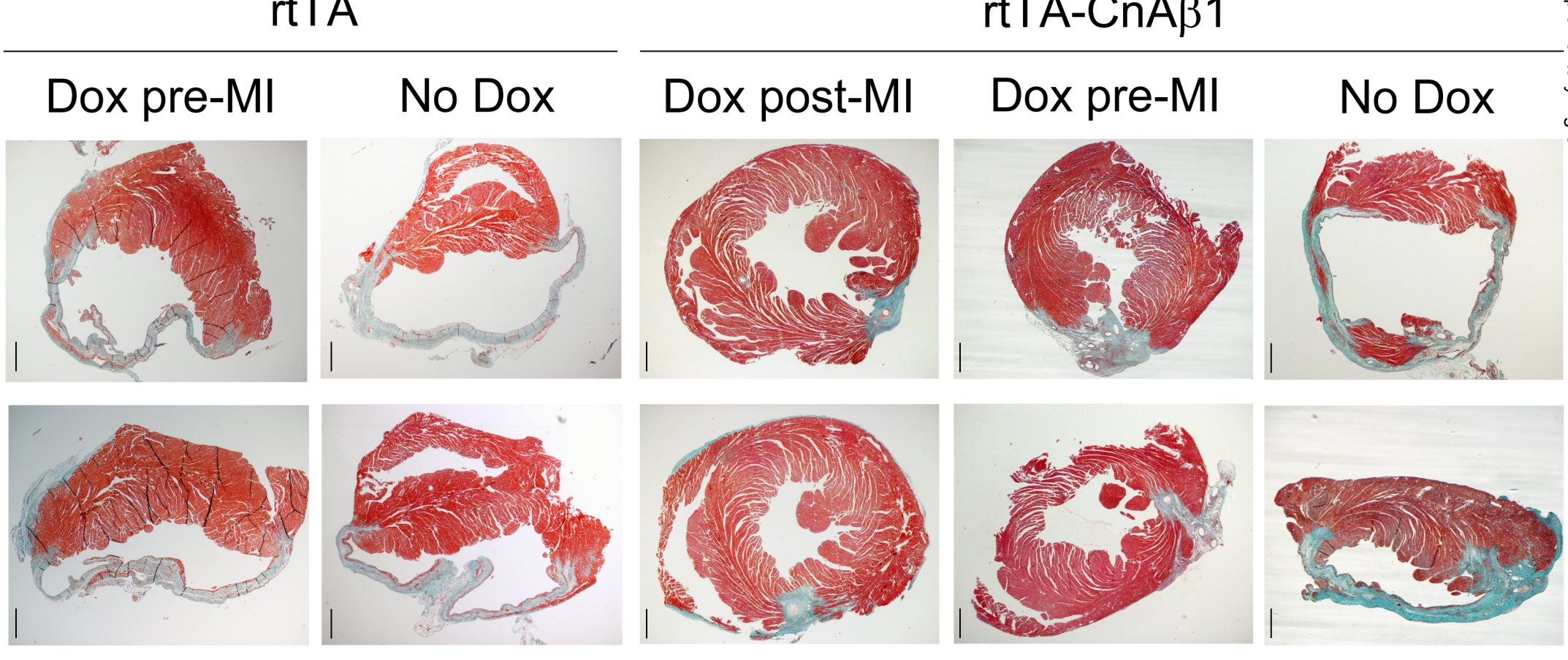
Figure S2. CnAβ1-overexpressing mice show shorter and thicker infarcts. This figure illustrates quantifications in Fig. 3. rtTA-CnAβ1 and rtTA mice, either treated or untreated with doxycycline, were sacrificed 28 days post-infarction. Hearts were excised, fixed and stained with Masson's trichrome protocol. Left ventricular sections show thicker and shorter scars in CnAβ1-overexpressing mice. Two images are shown per condition. Bar, 1 mm.

Figure S3. CnAβ1 overexpression results in a mild reduction in apoptotic cardiomyocytes 28 days post-infarction. A, B, Hearts from rtTA-CnAβ1 and rtTA mice were analysed by immunohistochemistry using Tunel staining. Fibroblasts (**A**) and cardiomyocytes (**B**) were identified by co-staining with anti-periostin I and anti-Tropoinin respectively. Results are expressed as percentage of apoptotic cells for each cell type ±SE.

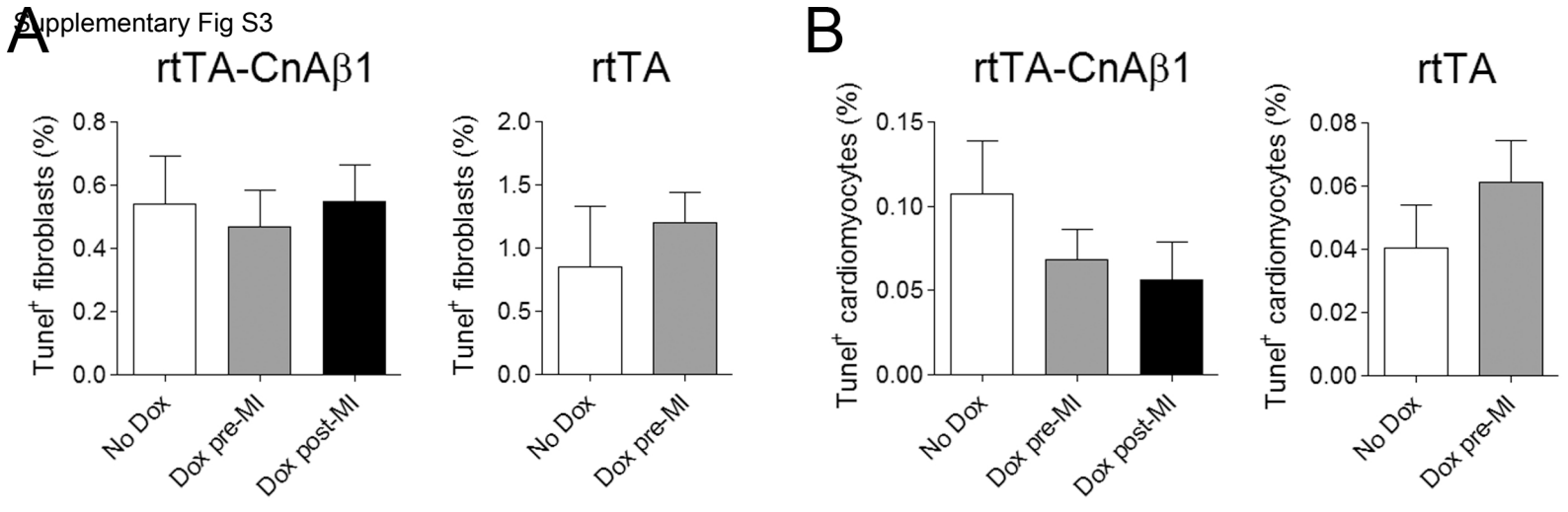
Supplementary Fig S1



López-Olañeta et al., Fig. S1


rtTA
rtTA-CnAβ1
Dox pre-MI
No Dox
Dox post-MI
Dox pre-MI
No Dox

Supplementary Fig S3



López-Olañeta et al., Fig. S3