Published in final edited form as:

Naunyn Schmiedebergs Arch Pharmacol. 2012 May ; 385(5): 443–453. doi:10.1007/s00210-011-0705-z.

$G\alpha_{i2}$ signaling: friend or foe in cardiac injury and heart failure?

Kuljeet Kaur,
Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109-0632, USA

Sergio Parra,
Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109-0632, USA

Rong Chen,
Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109-0632, USA

Raelene A. Charbeneau,
Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109-0632, USA

Susan M. Wade,
Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109-0632, USA

Patrick Y. Jay, and
Departments of Pediatrics and Genetics, Washington University School of Medicine, St. Louis, MO 63110, USA

Richard R. Neubig
Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, MI 48109-0632, USA

Department of Internal Medicine (Cardiovascular Medicine), The University of Michigan Medical School, 1150 W. Medical Center Drive 1303 MSRB III, Ann Arbor, MI 48109-0632, USA

Center for Chemical Genomics, The University of Michigan Medical School, Ann Arbor, MI 48109-0632, USA

Richard R. Neubig: rneubig@umich.edu

Abstract

Receptors coupled to G proteins have many effects on the heart. Enhanced signaling by $G\alpha_s$ and $G\alpha_q$ leads to cardiac injury and heart failure, while $G\alpha_{i2}$ signaling in cardiac myocytes can protect against ischemic injury and β-adrenergic-induced heart failure. We asked whether enhanced $G\alpha_{i2}$

Correspondence to: Richard R. Neubig, rneubig@umich.edu.

Electronic supplementary material The online version of this article (doi:10.1007/s00210-011-0705-z) contains supplementary material, which is available to authorized users.

signaling in mice could protect against heart failure using a point mutation in $G\alpha_{i2}$ (G184S), which prevents negative regulation by regulators of G protein signaling. Contrary to our expectation, it worsened effects of a genetic dilated cardiomyopathy (DCM) and catecholamine-induced cardiac injury. $G\alpha_{i2}^{G184S/+}$/DCM double heterozygote mice ($TG9^{+}G\alpha_{i2}^{G184S/+}$) had substantially decreased survival compared to DCM animals. Furthermore, heart weight/body weight ratios (HW/BW) were significantly greater in $TG9^{+}G\alpha_{i2}^{G184S/+}$ mice as was expression of natriuretic peptide genes. Catecholamine injury in $G\alpha_{i2}^{G184S/G184S}$ mutant mice produced markedly increased isoproterenol-induced fibrosis and collagen III gene expression vs WT mice. Cardiac fibroblasts from $G\alpha_{i2}^{G184S/G184S}$ mice also showed a serum-dependent increase in proliferation and ERK phosphorylation, which were blocked by pertussis toxin and a mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor. $G\alpha_{i2}$ signaling in cardiac myocytes protects against ischemic injury but enhancing $G\alpha_{i2}$ signaling overall may have detrimental effects in heart failure, perhaps through actions on cardiac fibroblasts.

Keywords

$G\alpha_{i2}$; RGS; Cardiac fibroblasts; Isoproterenol; Angiotensin

Introduction

The primary pharmacologic therapies that prolong life in congestive heart failure target beta adrenergic (βAR) and angiotensin (AT1) receptor signaling (Dzau 1992; Lechat et al. 1998; MacLellan 2000). The sympathetic and renin–angiotensin systems activate Gs- and Gq-type G proteins linked to cAMP and Ca^{++} signaling. There is abundant clinical and animal model data for the role of βAR and AT1R signaling and Gs and Gq to cause cardiac hypertrophy and heart failure (Schnabel and Bohm 1996).

In contrast to the deleterious effects of Gs and Gq signaling, there is significant literature on protection by Gi family G proteins, predominantly $G\alpha_{i2}$. Activation of many Gi-coupled receptors (e.g., adenosine, sphingosine-1-phosphate, estrogen, and opioid) can reduce ischemia– reperfusion injury. Loss of $G\alpha_{i2}$ function also worsens ischemic insults (DeGeorge et al. 2008), myocyte apoptosis (Chesley et al. 2000; DeGeorge et al. 2008), and heart failure [$G\alpha_{i2}$ knockout mice die prematurely in a model of βAR-induced heart failure (Foerster et al. 2003)]. These observations have prompted efforts to enhance signaling through $G\alpha_{i}$-family G proteins as a means to protect the heart.

Regulator of G protein signaling (RGS) proteins inhibit Gi and Gq protein signaling by increasing their GTPase activity (Hollinger and Hepler 2002). Blocking RGS action (Zhong and Neubig 2001; McEwen et al. 2002) could enhance protective effects of $G\alpha_{i2}$ in the heart. As a test of this idea, we used a point mutation (G184S) in $G\alpha_{i2}$ that blocks the binding of RGS proteins, prevents the RGS-mediated suppression of $G\alpha_{i2}$ signaling (Lan et al. 2000; Fu et al. 2004), and enhances $G\alpha_{i2}$ signaling in vitro and in vivo (Huang et al. 2006). We recently showed that $G\alpha_{i2}^{G184S}$ mutant mice have reduced myocardial injury in a Langendorff ischemia– reperfusion model (Waterson et al. 2011).

While $G\alpha_{i2}$ signaling in myocytes can protect the heart from injury and failure, increased $G\alpha_i$ signaling also, produces negative inotropic effects, functions in inflammatory mechanisms (Fan et al. 2005; Pero et al. 2007; Zarbock et al. 2007), and may increase fibroblast proliferation and fibrosis (Liu et al. 2006). Consequently, enhancing $G\alpha_i$ signaling as a means to cardioprotection may need to be selective to achieve the desired effect.

Here, we assess, in two distinct models of cardiac injury and heart failure, the effect of increased $G\alpha_{i2}$ signaling in mice with an RGS-insensitive $G\alpha_{i2}$ subunit. A mouse model of dilated cardiomyopathy (Tg9-DCM), like human heart failure, shows improved survival with angiotensin-converting enzyme inhibition and β_1AR blockade (Buerger et al. 2006) and worsens with abnormal glucose homeostasis (Hruz et al. 2008; Vyas et al. 2011). Second, isoproterenol injections are used to induce injury and subsequent heart failure (Ozaki et al. 2002). In both models, loss of RGS action at $G\alpha_{i2}$ led to worsened outcomes. In the DCM model, active mutant $G\alpha_{i2}$ produced early deaths and increased hypertrophy, and in the Iso-induced injury model, markedly increased fibrosis was seen. Furthermore, cardiac fibroblasts from the $G\alpha_{i2}^{G184S/\ G184S}$ mutant mice show a pertussis toxin sensitive increase in ERK activation and proliferation in vitro and enhanced collagen gene expression in vivo. Consequently, any manipulations to enhance $G\alpha_{i2}$ signaling in the heart should be selective for myocytes as $G\alpha_{i2}$ signaling in other tissues, such as fibroblasts may be detrimental.

Methods

Animals

Animal care was supervised by the University of Michigan Unit for Lab Animal Medicine and all animal study protocols were approved by the University Committee on Use and Care of Animals. Animals were maintained under a 12:12-h light–dark cycle with ad libitum access to standard chow and water. Mice with a genomic knock-in of the $G\alpha_{i2}^{G184S}$ RGS-insensitivity allele were described previously (Huang et al. 2006). All studies utilized mice crossed at least five times onto the appropriate genetic background (C57BL/6J or FVB/NJ).

The transgenic DCM model, which results from cardiac-specific over-expression of cre-recombinase, was previously described and the transgene is referred to as $TG9^+$ (Buerger et al. 2006). Heterozygous $G\alpha_{i2}^{G184S/+}$ females on an FVB/NJ background were crossed with $TG9^+$ (DCM) males also on an FVB/NJ background, yielding four genotypes: $G\alpha_{i2}^{+/+}$, $G\alpha_{i2}^{G184S/+}$, $TG9^+G\alpha_{i2}^{+/+}$, and $TG9^+G\alpha_{i2}^{G184S/+}$. For the isoproterenol studies, homozygous RGS-insensitive $G\alpha_{i2}^{G184S/G184S}$ mice and littermate controls (WT) mice were on the C57Bl/6J background.

Chronic drug treatment in the DCM model

Losartan (0.6 g/L) was added to drinking water at 3 weeks of age in $TG9^+G\alpha_{i2}^{+/+}$ vs $TG9^+G\alpha_{i2}^{G184S/+}$ mice. Doses were chosen from a previous report showing protective effects in a vascular model (Habashi et al. 2006). Survival was assessed up to 95 days for various genotypes and treatments.

Catecholamine-induced cardiac injury

Male, $G\alpha_{i2}^{G184S/\ G184S}$ and WT mice (14–16 weeks old) were treated with Iso (30 mg/kg) or saline via intraperitoneal injection daily for 7 days (Ozaki et al. 2002).

Cardiac tissue analysis

Animals were sacrificed at 10 weeks of age (DCM study) or 24 h after the last Iso or saline injection. Hearts were removed, washed with cold phosphate-buffered saline (PBS), and fixed for histology or frozen in liquid nitrogen for biochemical studies.

Fibrosis and cell size

Samples for histology were fixed in 10% formalin for 24 h, paraffin embedded, sectioned, stained with Masson's trichrome, imaged using light microscopy, and photographed (Zeiss Axioplan, Carl Zeiss IMT Corporation, Maple Grove, MN, USA). The percentage with collagen (blue) and without (red) was quantified using Adobe Photoshop (Dahab et al. 2004). For myocyte cross-sectional area, 10–20 randomly selected myoctyes per image were measured. Cells were outlined manually (total n=251–333 cells per condition) using the polygon selection tool ImageJ (http://rsb.info.nih.gov/nih-image). Due to the non-normal distribution of cell sizes, statistical analysis was done on the logarithm of cell area values.

mRNA analysis by qPCR

RNA isolated from frozen myocardial tissue using RNA STAT-60 reagent (Iso-Tex Diagnostics, Friendswood, TX, USA) was treated with DNase for 15 min at room temperature (Qiagen, Valencia, CA, USA) then quantitative PCR (qPCR) performed after first-strand complementary DNA (cDNA) synthesis (Taq-Man Kit, Applied Biosystems, Branchburg, NJ, USA). cDNA was then subjected to qPCR with SYBR green master mix (Stratagene, La Jolla, CA, USA) and 100 pM each of sense and antisense primer. Primers for genes relevant to cardiac hypertrophy (ANP, BNP, SMA, and Col III) and β-actin (an internal control) were described previously (Schoenfeld et al. 1998). PCR conditions were denaturation at 94°C and 10 min, then 40 cycles of denaturation at 94°C and 30 s, annealing at 60°C and 60 s, then extension at 72°C for 10 min. No-template controls and no-RT controls were run during each experiment to detect RNA and/or DNA contamination. Results are expressed as relative gene expression normalized against β-actin.

Adult cardiac fibroblast culture

Cardiac fibroblasts were isolated from 20- to 26-week-old female mice as described by O'Connell et al. (2007) with minor modifications. Briefly, after quick removal, hearts were washed in icecold PBS, then retrogradely perfused through the aorta for 5 min with calcium-free perfusion buffer (NaCl, 120.4 mM; KCl, 5 mM; $MgSO_4{\cdot}H_2O$, 1.2 mM; Na_2HPO_4, 0.6 mM; KH_2PO_4, 0.6 mM; Na–HEPES, 10 mM; $NaHCO_3$, 4.6 mM; taurine, 30 mM; glucose, 10 mM; and butanedione monoxime, 10 mM, pH 7.4). Hearts were then perfused for 10 min with the same buffer containing 0.5 mg/ml collagenase II (Worthington Biochemicals, Lakewood, NJ, USA, lot no. 46M9065). Hearts were removed from the apparatus and atria were discarded. Ventricles were suspended in perfusion buffer containing 10% serum and 1.25 μmol/L calcium to stop the digestion. Cells were separated from the tissue by gentle

pipetting of the digested heart using sterile plastic transfer pipettes. Samples were allowed to settle under gravity for 15 min to remove debris and myocytes. The remaining suspended fibroblasts were centrifuged at 300×g for 10 min and resuspended in Dulbecco's modified Eagle's medium supplemented with 1% penicillin/streptomycin, insulin/transferrin/selenium (ITS from Sigma), and 10% fetal bovine serum (full medium). Cells isolated by this method all stain positively for α-SMA (data not shown).

Fibroblast proliferation

Cardiac fibroblasts (passages 3–5) were plated in 24-well dishes (10^4/well) and allowed to grow in 10% "fetal bovine serum (FBS)-containing medium for the specified time in the presence or absence of pertussis toxin (PTX) (100 ng/ml). At the end of the growth period (1–4 days), medium was aspirated, cells were rinsed with Ca^{2+}/Mg^{2+} free PBS, and 150 μL of 0.05% trypsin was added to each well. Cells were incubated with trypsin at 37°C for 5 min and then shaken on a plate shaker at room temperature for 5 min. Then, 50 μL of FBS (100%) was added to stop trypsin action, and 200 μL of 10% formalin was added to fix cells. Cells were collected in microcentrifuge tubes, and 200 μL of this cell suspension was used for cell counting using a flow cytometer (Accuri Cytometers, Inc. Ann Arbor, MI, USA). To study the role of ERK, the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor PD 098059 (30 μM, Cayman Chemical, Ann Arbor, MI, USA) was added to the medium on day 1, and cells were counted on day 3.

ERK phosphorylation measurements

Western blotting was performed to assess ERK activation using a phospho-p44/ 42 Erk1/2 Thr202/Tyr204 antibody (no. 9101, Cell Signaling Technology, Inc., Danvers, MA, USA) and a total ERK antibody (no. 9102, Cell Signaling Technology, Inc., Danvers, MA, USA). Cells were seeded at an initial density of 2×10^5 cells/well in 12-well plates and grown in serumcontaining medium for 24 h in the absence or presence of PTX. Cells were washed with cold PBS and lysed with RIPA buffer (Tris, 25 mM; NaCl, 150 mM; NP-40, 1%; sodium deoxycholate, 1%; sodium dodecyl sulfate, 0.1%). The cell lysates (20 μg) were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunostained, then visualized using enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA). The band intensities (p42+p44) were quantified densitometrically, and ERK phosphorylation was expressed as the intensity of the pERK bands normalized to the intensity of the total ERK bands.

Statistical analyses

Comparisons of individual group means used a two-tailed Student's *t* test. One- or two-way analysis of variance (ANOVA) with Bonferroni post-test was used to compare multiple data sets. Survival curves were compared by log-rank (Mantel–Cox) test. All statistical calculations were done using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA), and $p< 0.05$ was considered significant.

Results

Effect of $G\alpha_{i2}^{G184S/+}$ mutation on survival in DCM mice

The TG9 DCM model leads to the development of congestive heart failure and death (Buerger et al. 2006). As seen previously, the maximum life span for $TG9^{+}G\alpha_{i2}^{+/+}$ males was 13.5 weeks (95 days, Fig. 1a), and for females, it was about 12 weeks (85 days, Fig. 1b). There were no deaths in non-TG9 mice during the 95-day duration of the study, regardless of their $G\alpha_{i2}$ genotype. Combining the RGS-insensitive $G\alpha_{i2}$ mutation (Huang et al. 2006) with the $TG9^{+}$ mutation significantly worsened survival. $TG9^{+}G\alpha_{i2}^{G184S/+}$ male mice survived for maximum of 88 days, and females survived for only 79 days (Fig. 1a and Table 1, $p<0.05$ for male and Fig. 1b, $p< 0.001$ for female).

Effect of the $G\alpha_{i2}^{G184S/+}$ mutation on cardiac hypertrophy in $TG9^{+}$ mice

At 10 weeks of age (i.e., prior to any deaths), male DCM mice with WT $G\alpha_{i2}$ showed a modest increase in HW/BW, which was markedly greater in the double-mutant TG9+ $G\alpha_{i2}^{G184S/+}$ mice (Fig. 2a, $p<0.001$, two-way ANOVA vs $G\alpha_{i2}^{G184S/+}$ controls and $p<0.01$ vs DCM $G\alpha_{i2}^{+/+}$ mice). Consistent with the earlier onset of heart failure, 10-week-old $TG9^{+}G\alpha_{i2}^{G184S/+}$ male mice also showed a significant increase in lung weight to body weight ratio (LW/BW, Fig. 2b) compared to both $TG9^{-}$ controls ($p<0.01$) and $TG9^{+}$ mice with WT $G\alpha_{i2}$ ($p<0.01$).

Female mice of both $G\alpha_{i2}$ genotypes showed significant hypertrophy at 10 weeks, 20.2±4.1% and 40.1±3.2% increase in HW/BW, respectively, for DCM $G\alpha_{i2}$ and $G\alpha_{i2}^{G184S/+}$ compared to their non-DCM controls (Fig. 2c, $p<0.001$). The difference between DCM $G\alpha_{i2}^{G184S/+}$ and DCM $G\alpha_{i2}^{+/+}$ was not statistically significant most likely due to the more advanced progression of heart failure in females at 10 weeks. $TG9^{+}G\alpha_{i2}^{++}$ and $TG9^{+}G\alpha_{i2}^{G184S/+}$ female mice both had significant increases in LW/BW (17.0± 7.9% vs 32.2±4.5%, $p<0.05$ and $p<0.001$, respectively).

Effect of $G\alpha_{i2}^{G184S/+}$ mutation on marker gene expression and fibrosis

Heterozygous $G\alpha_{i2}^{G184S/+}$ mutant mice had normal expression of all four of the marker genes studied (ANP, BNP, SMA, and Col III, Figs. ESM1 and ESM2), while DCM mice had marked increases. As seen for HW/BW, male DCM mice carrying the $G\alpha_{i2}^{G184S/+}$ mutation showed a greater increase in ANP and BNP expression at 10 weeks vs DCM alone (ANP, $p<0.001$; BNP, $p< 0.01$). In contrast, DCM females showed major increases in natriuretic peptide gene expression, but there was no further effect of the $G\alpha_{i2}^{G184S/+}$ mutation (Fig. ESM2). The effect of the $G\alpha_{i2}^{G184S/+}$ mutation appears to have been overshadowed by the earlier onset of heart failure in the females.

Both male and female DCM mice exhibited a modest increase in fibrosis compared to non-DCM mice, but there was no augmentation when they were carrying the $G\alpha_{i2}^{G184S/+}$ mutation vs WT $G\alpha_{i2}$ (data not shown). This result was in agreement with the levels of expression seen for Col III (Figs. ESM1D and ESM2D).

Effect of losartan treatment

To assess signaling pathways that might be involved in worsened cardiac failure with the $G\alpha_{i2}^{G184S/+}$ mutation, we tested pharmacological blockade of angiotensin II signaling. Female mice, chosen due to the greater effect of the $G\alpha_{i2}^{G184S/+}$ mutation, were given drinking water containing losartan starting at 3 weeks of age. Losartan treatment significantly improved survival. The difference between the control and losartan treatment groups was highly significant for both genotypes (Fig. 3a, $p<0.001$). The losartan-induced change in survival (4.85±0.54 days for $TG9_{+}G\alpha_{i2}^{G184S/+}$ vs 3.51±0.30 days for $TG9_{+}G\alpha_{i2}^{+/+}$) was significantly greater for double-mutants compared to the $TG9^{+}/G\alpha_{i2}^{+/+}$ mice (Fig. 3b, $p<0.05$).

Catecholamine injury model: effect of $G\alpha_{i2}$ G184S mutation

Seven days of Iso treatment resulted in one death among the nine WT mice (on day 4) while three of the nine RGS-insensitive $G\alpha_{i2}^{G184S/G184S}$ mice (or G184S/G184S) mice died on the second day of Iso treatment. As reported previously (Huang et al. 2006), there was a modestly greater HW/BW in homozygous $G\alpha_{i2}^{G184S/G184S}$ mice than in WT mice ($p<0.01$, unpaired, *t* test). Iso treatment increased HW/BW in both WT and mutant mice (Fig. 4a). There were significant effects of both treatment ($p<0.001$) and genotype ($p<0.05$) by two-way ANOVA and a Bonferroni post-test showed significant Iso effects (Fig. 5a, $p<0.05$) for both WT and $G\alpha_{i2}^{G184S/G184S}$ mice.

Since heart weight is determined by both myocyte and non-myocyte elements, we also assessed myocyte size (Fig. 4b, c), which was 24% greater in the hearts from saline-treated mutant mice (262 μm^2) compared to those from WT mice (211 μm^2), consistent with the 30% greater HW/BW. Iso treatment increased myocyte size in WT mice (293 vs 211 μm^2), while for $G\alpha_{i2}^{G184S/G184S}$, cell size was unchanged (261 vs 262 μm^2) despite an increase in HW/ BW of 32%. The lack of effect on myocyte size for mutants in the face of increased HW/BW suggests a role of non-myocyte elements in the Iso-induced cardiac hypertrophy in mutant mice (see below).

Effect of $G\alpha_{i2}^{G184S}$ mutation on marker gene expression and fibrosis

Mutant mice had significantly higher basal expression of BNP (Fig. 5, $p<0.05$). ANP in saline-treated $G\alpha_{i2}^{G184S/G184S}$ mice also was significantly greater (750±290% of WT values, $p< 0.05$, by non-paired *t* test). Iso treatment significantly increased the expression of ANP and SMA in both WT and $G\alpha_{i2}$ mutant mice (Fig. 5, $p<0.05$); however, the magnitude was not different between the WT and mutant mice.

Mutants, however, showed a markedly increased expression of Col III messenger RNA (mRNA; 725±172%, $p<0.01$) after Iso with no effect in WT mice. The difference between WT and $G\alpha_{i2}^{G184S/G184S}$ mice after Iso treatment was also highly significant ($p<0.001$, Fig. 5).

Effect of $G\alpha_{i2}^{G184S}$ mutation on fibrosis

To understand the functional relevance of this increase in Col III mRNA expression, we analyzed cardiac fibrosis by trichrome staining (Dahab et al. 2004). Consistent with the gene

expression results, there was a marked increase in fibrosis in mutant mice after Iso treatment ($p<0.01$), and this level of fibrosis was much greater than that seen in WT hearts after Iso treatment ($p<0.001$, Fig. 6a, b). The effect of Iso treatment on fibrosis in WT mice (264±58% of saline), was not statistically significant.

Effect of $G\alpha_{i2}^{G184S}$ mutation on cardiac fibroblast proliferation

We examined cardiac fibroblasts and found a marked difference in their growth properties under normal growth conditions (i.e., 10% FBS). Cardiac fibroblast were isolated from three WT and three $G\alpha_{i2}^{G184S/G184S}$ mice and mutant cells grew significantly faster than WT (Fig. 7a, b); 104±45% more at 3 days in culture ($p<0.05$). There was no difference between WT and mutant fibroblast proliferation in low serum medium (Table ESM1). Fibroblast numbers were significantly decreased by PTX treatment for both mutant ($p<0.001$) and WT ($p<0.05$). After PTX, the difference in the fibroblast numbers between WT and mutant was not significant, so ongoing signaling through PTX-sensitive mechanisms (presumably via $G\alpha_{i2}$ coupled receptors) contributes to increased proliferation/survival of the $G\alpha_{i2}^{G184S/G184S}$ cardiac fibroblasts.

Effect of $G\alpha_{i2}^{G184S}$ mutation on cardiac fibroblast ERK signaling

Mutant cardiac fibroblasts grown in serum-containing medium had increased phospho-ERK levels (207±34% of WT, Fig. 8b, $p<0.05$). This increased ERK phosphorylation was abolished by PTX treatment ($p<0.05$) and was absent in mutant cardiac fibroblast grown in serum free medium (Fig. 8c). To determine whether this might contribute to the enhanced proliferation/survival of $G\alpha_{i2}^{G184S/G184S}$ mutant fibroblasts, we evaluated treatment with the MEK inhibitor, PD 98059, which significantly decreased the number of mutant fibroblasts (Fig. 8d, $p<0.05$), while it had no effect on WT fibroblasts.

Discussion

The unclear significance of $G\alpha_{i2}$ signaling in myocardial injury complicates efforts to modulate its function as a therapeutic strategy. Early reports showing increased $G\alpha_{i2}$ expression in heart failure patients (Feldman et al. 1988) did not differentiate between cause and effect regarding complications of heart failure. Recent studies on protective roles of $G\alpha_{i2}$ signaling (Chesley et al. 2000; Foerster et al. 2003; DeGeorge et al. 2008) studied myocytes or used in vivo genetic manipulations specific to myocytes perhaps missing a role for other cell types. Here, we used a $G\alpha_{i2}^{G184S}$ RGS-insensitive mouse model of enhanced $G\alpha_{i2}$ signaling cardiac pathophysiology. We recently showed that these mice are protected from myocardial ischemia/reperfusion injury (Waterson et al. 2011). However, in heart failure models examined here, detrimental effects were seen. $G\alpha_{i2}^{G184S}$ mutant mice show early mortality in the DCM model, which is partially ameliorated by angiotensin receptor blockade. In addition, they have markedly increased fibrosis after Iso-induced injury in vivo and increased cardiac fibroblast ERK activity and cell proliferation. These results further emphasize the growing realization that signaling in fibroblasts, as well as that in myocytes, may be key to understanding cardiac pathophysiology (Baudino et al. 2006).

The best evidence for a protective role of $G\alpha_i$ in heart failure comes from studies of the β2-AR. Overexpression of the β1-AR causes severe heart failure (Engelhardt et al. 1999) but similar expression of the β2-AR does not (Foerster et al. 2003). This has been attributed to a Gi-dependent anti-apoptotic effect in cardiac myocytes (Chesley et al. 2000; DeGeorge et al. 2008). Knockout of $G\alpha_{i2}$ (Foerster et al. 2003; DeGeorge et al. 2008) or pertussis toxin treatment of myocytes in vitro (Chesley et al. 2000) reverses the apparent protection afforded by Gi signaling. Consequently, the adverse outcomes seen here with RGSi mutation-induced enhancement of $G\alpha_{i2}$ signaling were surprising.

Fibroblasts are key cells in cardiac remodeling, but we are not able to discriminate with this study whether they initiatiated the pathophysiological response or if they were simply responding to the myocyte dysfunction and death occasioned by cardiac injury. Future studies will be needed to determine that. In the context of the $G\alpha_{i2}^{G184S}$ mice, catecholamine-induced cardiac injury did lead to more fibrosis, while in the DCM model the mutation seems to primarily facilitate hypertrophy. Although there was no a direct link between the Iso-induced fibrosis and the decline in DCM survival, end points for both models show a common detrimental role of enhanced $G\alpha_{i2}$ signaling.

The fibrosis after high-dose Iso treatment is consistent with known Gi signaling in cardiac fibroblasts by thrombin (Sabri et al. 2002) and lysophosphatidic acid (LPA) (Epperson et al. 2009). RGS-insensitive $G\alpha_{i2}$ MEFs show increased LPA-stimulated ERK and Akt phosphorylation (Huang et al. 2006), so LPA may be the serum factor causing the enhanced our Gi- and ERK-pathway-dependent proliferation. The Gi-linked LPA_3 receptor induces proliferation and collagen synthesis in cardiac fibroblasts (Chen et al. 2006), and LPA levels in serum are markedly increased after myocardial infarction (Chen et al. 2003).

The modest increases in HW/BW in the $G\alpha_{i2}^{G184S/G184S}$ mice [Huang et al. (2006) and Fig. 4a] could have been either physiologic hypertrophy, protective against heart failure, or pathologic hypertrophy that would worsen heart failure. Our present data now point to pathological hypertrophy. Earlier death in the DCM transgenic heart failure model provides one of the hallmarks of pathological hypertrophy (i.e., exacerbation of other hypertrophic insults). Furthermore, the increased expression of “hypertrophy genes” such as BNP and ANP is characteristic of pathologic hypertrophy. The $G\alpha_{i2}^{G184S/G184S}$ homozygotes showed increases in natriuretic peptide expression at baseline, while both Iso and DCM insults led to substantially higher hypertrophy gene expression in the mutants.

Angiotensin AT1 receptors may contribute in the DCM model. Losartan produces improved survival of DCM mice, and this was significantly greater for mice with the $G\alpha_{i2}^{G184S/+}$ mutant (Fig. 3b, c). In addition to G_q signaling, AT1 receptors can activate Raf-1 and ERK in liver epithelial cells (Tsygankova et al. 1998) and increase proliferation and ERK activation with cardiac fibroblasts (Zou et al. 1998) via PTX-sensitive signals. Enhanced AT1 receptor activation of $G\alpha_{i2}$, perhaps in fibroblasts, may contribute to the worsened heart failure in $G\alpha_{i2}^{G184S}$ mice.

Our results raise a number of questions for future consideration. First, which RGS proteins control $G\alpha_{i2}$ signaling relevant to the worsened injury, and do they differ from those in

myocytes that suppress $G\alpha_{i2}$-mediated protective effects seen in the ischemia-reperfusion model (Waterson et al. 2011)? If so, one could target the latter pharmacologically (Neubig and Siderovski 2002; Riddle et al. 2005; Blazer et al. 2011) to improve outcomes in myocardial infarction with less risk of complications from subsequent heart failure. Second, which receptors signal through $G\alpha_{i2}$ to induce the worsened heart failure and fibrosis? Identification of these receptors could reveal potential targets of therapeutic intervention. While angiotensin blockade improves survival in the DCM model, we cannot say that this is a direct effect of AT1R on $G\alpha_{i2}$ as it could be a downstream action such as from released chemokines, S1P, etc., which act through Gicoupled pathways. Studies to assess these questions are ongoing.

In summary, we asked whether the whole-body knock-in of the RGS-insensitive $G\alpha_{i2}^{G184S}$ mutation could protect against cardiac injury and heart failure. We show, however, that in two animal models, RGS proteins through their action on $G\alpha_{i2}$ appear to play a protective role perhaps in part by reducing ERK activity and proliferation of cardiac fibroblasts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study supported by National Institutes of Health grant R01-GM39561 (R.R.N.) and the University of Michigan Comprehensive Cancer Center (National Institutes of Health grant P30-CA46592). PYJ is a Scholar of the Child Health Research Center of Excellence in Developmental Biology at Washington University School of Medicine (National Institutes of Health K12-HD001487).

References

Baudino TA, Carver W, Giles W, Borg TK. Cardiac fibroblasts: friend or foe? Am J Physiol Heart Circ Physiol. 2006; 291:H1015–H1026. [PubMed: 16617141]

Blazer LL, Zhang H, Casey EM, Husbands SM, Neubig RR. A nanomolar-potency small molecule inhibitor of regulator of G protein signaling (RGS) proteins. Biochemistry. 2011; 50:3181–3192. [PubMed: 21329361]

Buerger A, Rozhitskaya O, Sherwood MC, Dorfman AL, Bisping E, Abel ED, Pu WT, Izumo S, Jay PY. Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase. J Card Fail. 2006; 12:392–398. [PubMed: 16762803]

Chen X, Yang XY, Wang ND, Ding C, Yang YJ, You ZJ, Su Q, Chen JH. Serum lysophosphatidic acid concentrations measured by dot immunogold filtration assay in patients with acute myocardial infarction. Scand J Clin Lab Invest. 2003; 63:497–503. [PubMed: 14743959]

Chen J, Han Y, Zhu W, Ma R, Han B, Cong X, Hu S, Chen X. Specific receptor subtype mediation of LPA-induced dual effects in cardiac fibroblasts. FEBS Lett. 2006; 580:4737–4745. [PubMed: 16890224]

Chesley A, Lundberg MS, Asai T, Xiao RP, Ohtani S, Lakatta EG, Crow MT. The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3′-kinase. Circ Res. 2000; 87:1172–1179. [PubMed: 11110775]

Dahab GM, Kheriza MM, El-Beltagi HM, Fouda AM, El-Din OA. Digital quantification of fibrosis in liver biopsy sections: description of a new method by Photoshop software. J Gastroenterol Hepatol. 2004; 19:78–85. [PubMed: 14675247]

DeGeorge BR Jr, Gao E, Boucher M, Vinge LE, Martini JS, Raake PW, Chuprun JK, Harris DM, Kim GW, Soltys S, Eckhart AD, Koch WJ. Targeted inhibition of cardiomyocyte Gi signaling enhances

susceptibility to apoptotic cell death in response to ischemic stress. Circulation. 2008; 117:1378–1387. [PubMed: 18316484]

Dzau VJ. Autocrine and paracrine mechanisms in the pathophysiology of heart failure. Am J Cardiol. 1992; 70:4C–11C.

Engelhardt S, Hein L, Wiesmann F, Lohse MJ. Progressive hypertrophy and heart failure in beta1-adrenergic receptor transgenic mice. Proc Natl Acad Sci U S A. 1999; 96:7059–7064. [PubMed: 10359838]

Epperson SA, Brunton LL, Ramirez-Sanchez I, Villarreal F. Adenosine receptors and second messenger signaling pathways in rat cardiac fibroblasts. Am J Physiol Cell Physiol. 2009; 296:C1171–C1177. [PubMed: 19244482]

Fan H, Zingarelli B, Peck OM, Teti G, Tempel GE, Halushka PV, Spicher K, Boulay G, Birnbaumer L, Cook JA. Lipopolysaccharide-and gram-positive bacteria-induced cellular inflammatory responses: role of heterotrimeric Galpha(i) proteins. Am J Physiol Cell Physiol. 2005; 289:C293–C301. [PubMed: 15788486]

Feldman AM, Cates AE, Veazey WB, Hershberger RE, Bristow MR, Baughman KL, Baumgartner WA, Van Dop C. Increase of the 40,000-mol wt pertussis toxin substrate (G protein) in the failing human heart. J Clin Invest. 1988; 82:189–197. [PubMed: 2839545]

Foerster K, Groner F, Matthes J, Koch WJ, Birnbaumer L, Herzig S. Cardioprotection specific for the G protein Gi2 in chronic adrenergic signaling through beta 2-adrenoceptors. Proc Natl Acad Sci U S A. 2003; 100:14475–14480. [PubMed: 14612574]

Fu Y, Zhong H, Nanamori M, Mortensen RM, Huang X, Lan K, Neubig RR. RGS-insensitive G-protein mutations to study the role of endogenous RGS proteins. Methods Enzymol. 2004; 389:229–243. [PubMed: 15313569]

Habashi JP, Judge DP, Holm TM, Cohn RD, Loeys BL, Cooper TK, Myers L, Klein EC, Liu G, Calvi C, Podowski M, Neptune ER, Halushka MK, Bedja D, Gabrielson K, Rifkin DB, Carta L, Ramirez F, Huso DL, Dietz HC. Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. Science. 2006; 312:117–121. [PubMed: 16601194]

Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. Pharmacol Rev. 2002; 54:527–559. [PubMed: 12223533]

Hruz PW, Yan Q, Struthers H, Jay PY. HIV protease inhibitors that block GLUT4 precipitate acute, decompensated heart failure in a mouse model of dilated cardiomyopathy. FASEB J. 2008; 22:2161–2167. [PubMed: 18256305]

Huang X, Fu Y, Charbeneau RA, Saunders TL, Taylor DK, Hankenson KD, Russell MW, D'Alecy LG, Neubig RR. Pleiotropic phenotype of a genomic knock-in of an RGS-insensitive G184S Gnai2 allele. Mol Cell Biol. 2006; 26:6870–6879. [PubMed: 16943428]

Lan KL, Zhong H, Nanamori M, Neubig RR. Rapid kinetics of regulator of G-protein signaling (RGS)-mediated Galphai and Galphao deactivation. Galpha specificity of RGS4 AND RGS7. J Biol Chem. 2000; 275:33497–33503. [PubMed: 10942773]

Lechat P, Packer M, Chalon S, Cucherat M, Arab T, Boissel JP. Clinical effects of beta-adrenergic blockade in chronic heart failure: a meta-analysis of double-blind, placebo-controlled, randomized trials. Circulation. 1998; 98:1184–1191. [PubMed: 9743509]

Liu X, Sun SQ, Hassid A, Ostrom RS. cAMP inhibits transforming growth factor-beta-stimulated collagen synthesis via inhibition of extracellular signal-regulated kinase 1/2 and Smad signaling in cardiac fibroblasts. Mol Pharmacol. 2006; 70:1992–2003. [PubMed: 16959941]

MacLellan WR. Advances in the molecular mechanisms of heart failure. Curr Opin Cardiol. 2000; 15:128–135. [PubMed: 10952417]

McEwen DP, Gee KR, Kang HC, Neubig RR. Fluorescence approaches to study G protein mechanisms. Methods Enzymol. 2002; 344:403–420. [PubMed: 11771399]

Neubig RR, Siderovski DP. Regulators of G-protein signalling as new central nervous system drug targets. Nat Rev Drug Discov. 2002; 1:187–197. [PubMed: 12120503]

O'Connell TD, Rodrigo MC, Simpson PC. Isolation and culture of adult mouse cardiac myocytes. Meth Mol Biol. 2007; 357:271–296.

Ozaki M, Kawashima S, Yamashita T, Hirase T, Ohashi Y, Inoue N, Hirata K, Yokoyama M. Overexpression of endothelial nitric oxide synthase attenuates cardiac hypertrophy induced by chronic isoproterenol infusion. Circ J. 2002; 66:851–856. [PubMed: 12224825]

Pero RS, Borchers MT, Spicher K, Ochkur SI, Sikora L, Rao SP, Abdala-Valencia H, O'Neill KR, Shen H, McGarry MP, Lee NA, Cook-Mills JM, Sriramarao P, Simon MI, Birnbaumer L, Lee JJ. Galphai2-mediated signaling events in the endothelium are involved in controlling leukocyte extravasation. Proc Natl Acad Sci U S A. 2007; 104:4371–4376. [PubMed: 17360531]

Riddle EL, Schwartzman RA, Bond M, Insel PA. Multi-tasking RGS proteins in the heart: the next therapeutic target? Circ Res. 2005; 96:401–411. [PubMed: 15746448]

Sabri A, Short J, Guo J, Steinberg SF. Protease-activated receptor-1-mediated DNA synthesis in cardiac fibroblast is via epidermal growth factor receptor transactivation: distinct PAR-1 signaling pathways in cardiac fibroblasts and cardiomyocytes. Circ Res. 2002; 91:532–539. [PubMed: 12242272]

Schnabel P, Bohm M. Heterotrimeric G proteins in heart disease. Cell Signal. 1996; 8:413–423. [PubMed: 8958443]

Schoenfeld JR, Vasser M, Jhurani P, Ng P, Hunter JJ, Ross J Jr, Chien KR, Lowe DG. Distinct molecular phenotypes in murine cardiac muscle development, growth, and hypertrophy. J Mol Cell Cardiol. 1998; 30:2269–2280. [PubMed: 9925364]

Tsygankova OM, Peng M, Maloney JA, Hopkins N, Williamson JR. Angiotensin II induces diverse signal transduction pathways via both Gq and Gi proteins in liver epithelial cells. J Cell Biochem. 1998; 69:63–71. [PubMed: 9513047]

Vyas AK, Yang KC, Woo D, Tzekov A, Kovacs A, Jay PY, Hruz PW. Exenatide improves glucose homeostasis and prolongs survival in a murine model of dilated cardiomyopathy. PLoS One. 2011; 6:e17178. [PubMed: 21359201]

Waterson RE, Thompson CG, Mabe NW, Kaur K, Talbot JN, Neubig RR, Rorabaugh BR. G{alpha}i2-mediated protection from ischaemic injury is modulated by endogenous RGS proteins in the mouse heart. Cardiovasc Res. 2011; 91:45–52. [PubMed: 21349876]

Zarbock A, Deem TL, Burcin TL, Ley K. Galphai2 is required for chemokine-induced neutrophil arrest. Blood. 2007; 110:3773–3779. [PubMed: 17699741]

Zhong H, Neubig RR. Regulator of G protein signaling proteins: novel multifunctional drug targets. J Pharmacol Exp Ther. 2001; 297:837–845. [PubMed: 11356902]

Zou Y, Komuro I, Yamazaki T, Kudoh S, Aikawa R, Zhu W, Shiojima I, Hiroi Y, Tobe K, Kadowaki T, Yazaki Y. Cell type-specific angiotensin II-evoked signal transduction pathways: critical roles of Gbetagamma subunit, Src family, and Ras in cardiac fibroblasts. Circ Res. 1998; 82:337–345. [PubMed: 9486662]

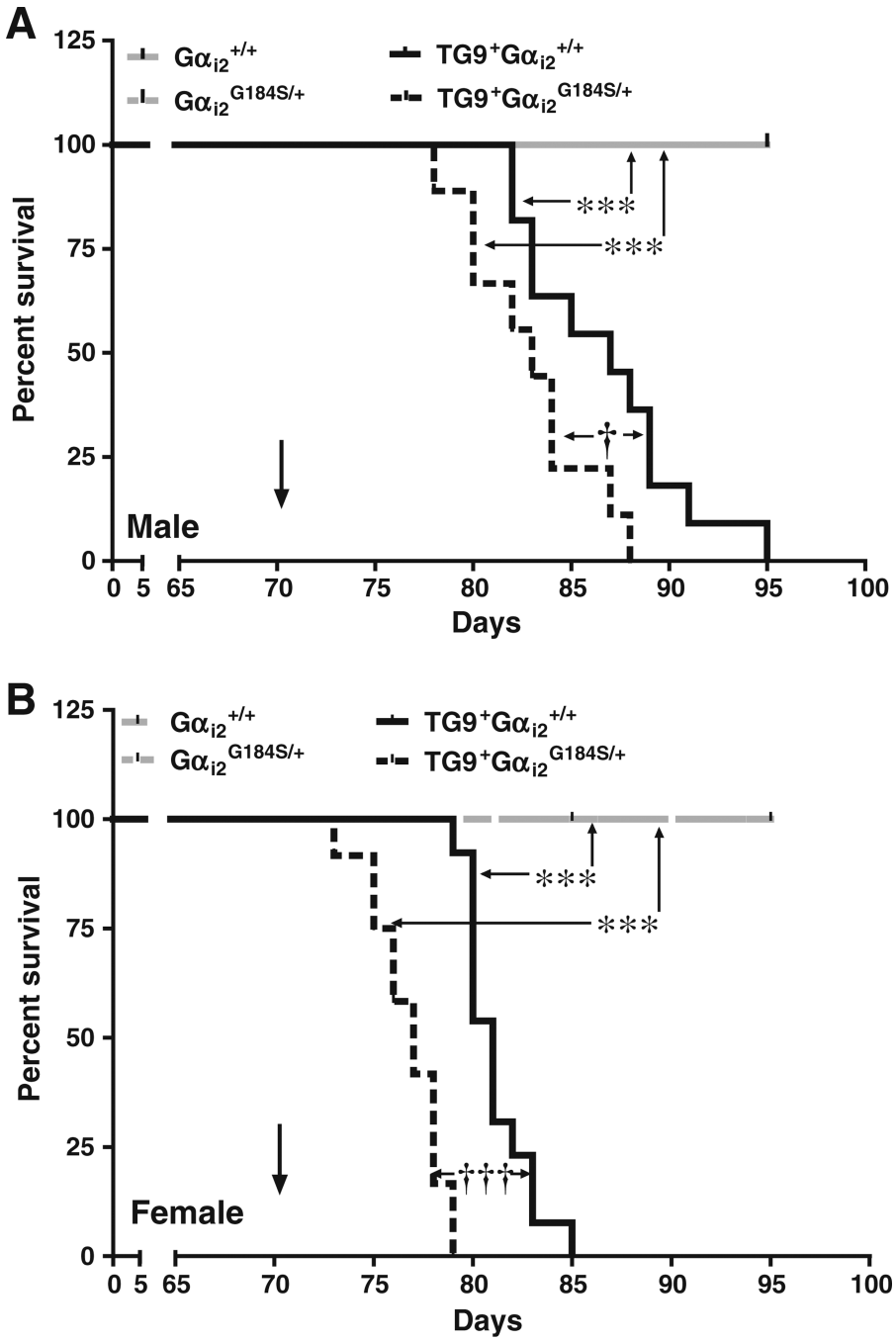

Fig. 1.

Effect of RGS-insensitive Gα$_{i2}^{G184S/+}$ mutation on survival of DCM mice. Kaplan–Meier survival curves in **a** males and **b** females for Gα$_{i2}^{+/+}$ (WT); Gα$_{i2}^{G184S/+}$; TG9^{+}Gα$_{i2}^{+/+}$ (DCM); TG9^{+}Gα$_{i2}^{G184S/+}$ (DCM/Gα$_{i2}^{G184S/+}$) were compared by log-rank (Mantel–Cox) test. ***p<0.001 compared to respective TG9-control. $^{\dagger}p$<0.05; $^{\dagger\dagger\dagger}p$<0.001 compared to respective Gα$_{i2}^{+/+}$control. n=9–13. *Arrows* indicate the time at which tissues were collected in subsequent studies

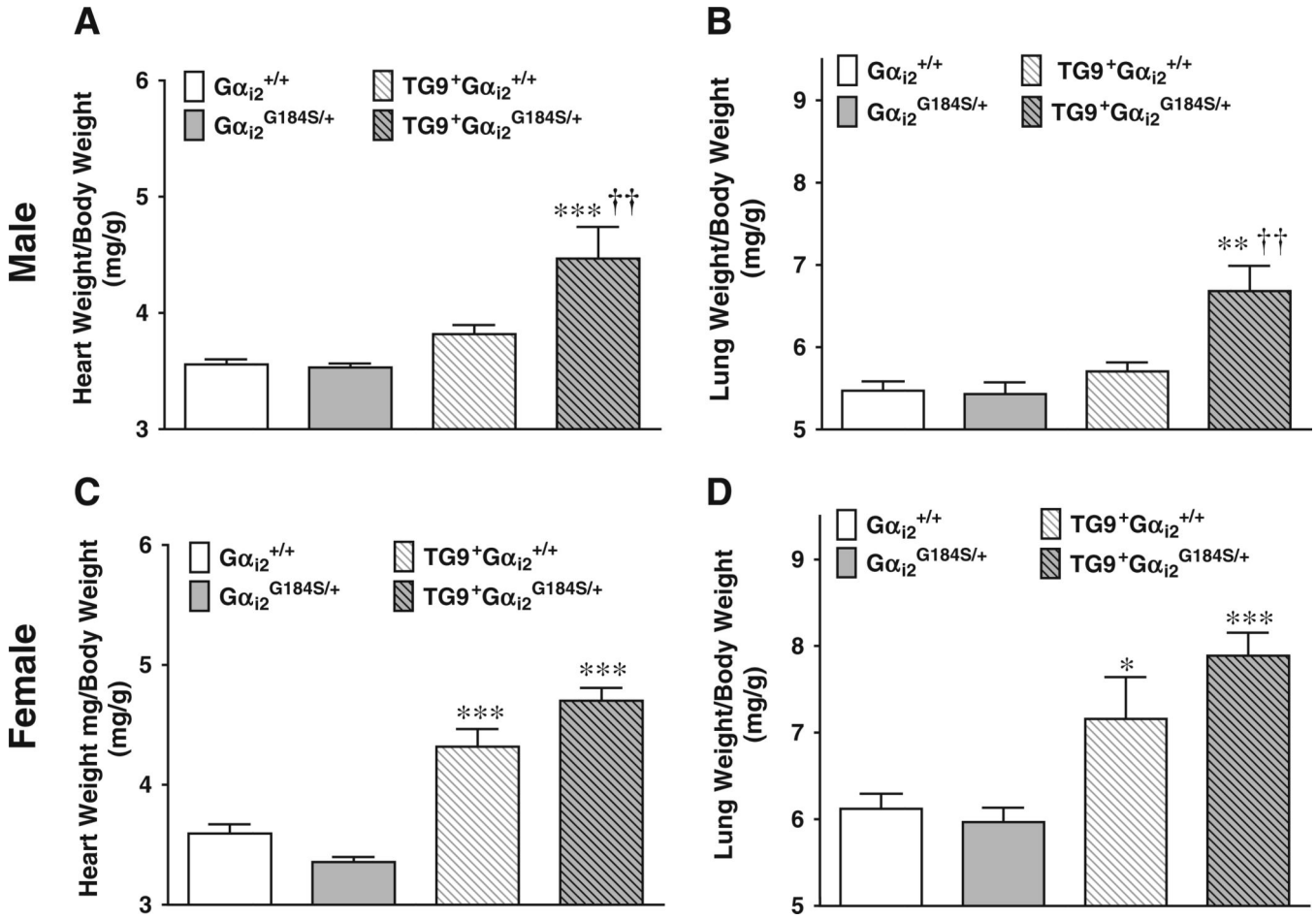

Fig. 2.

Effect of $G\alpha_{i2}^{G184S/+}$ on the heart weight and lung weight in DCM mice. **a, c** HW/BW, **b, d** LW/BW in males (**a, b**) and females (**c, d**) at 10 weeks of age. Values are mean±standard error of the mean, n=4–6 in each group. *p<0.05 compared to non-DCM-control. $^{\dagger}p$< 0.05 compared to $G\alpha_{i2}^{+/+}$. Differences were determined by two-way ANOVA with Bonferroni post-test

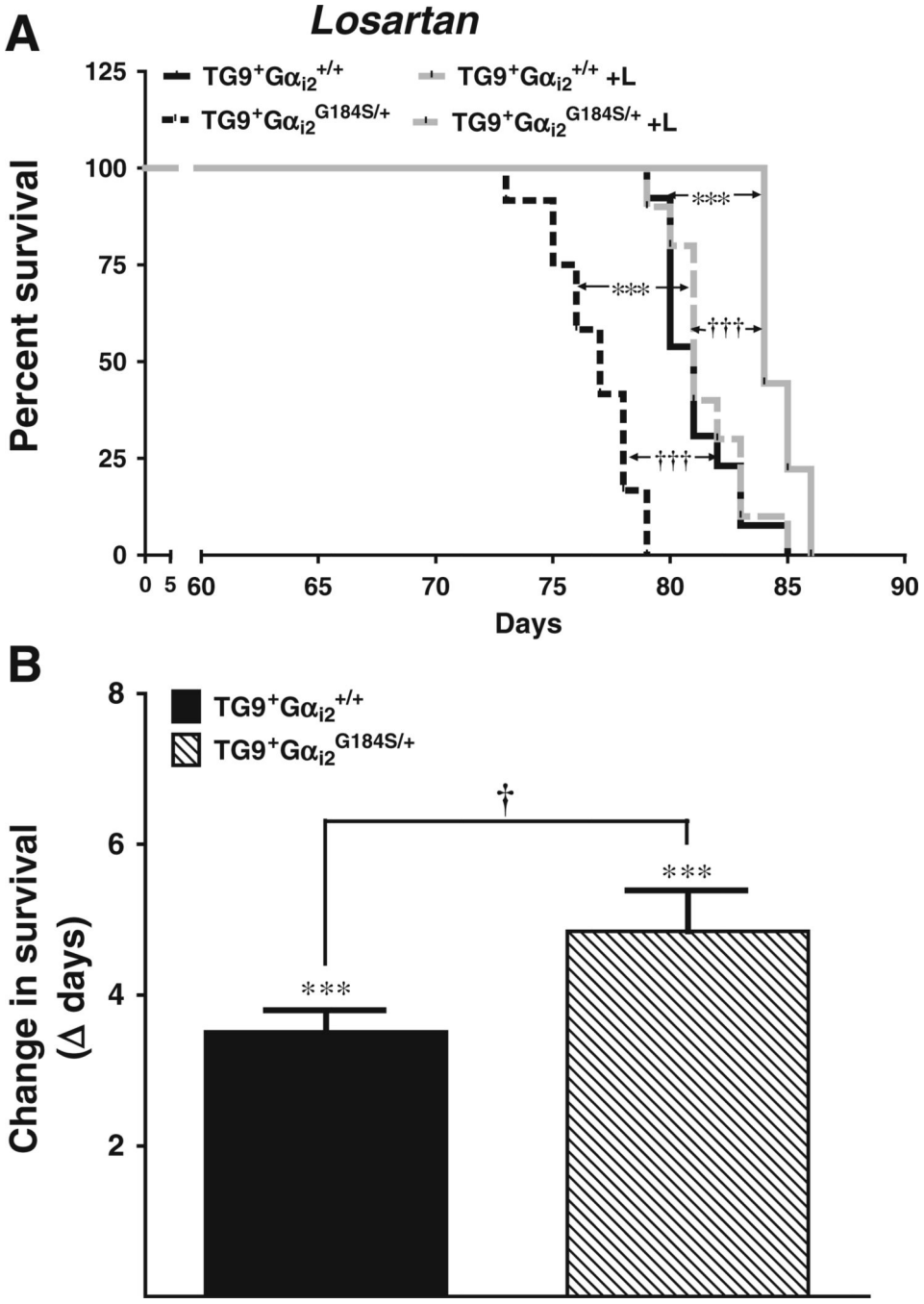

Fig. 3.

Effect of losartan treatment on survival. Losartan (**a**) was included in drinking water (see "Methods"). ***p<0.001 for effect of drug treatment compared to non-drug control. $^{†††}p$<0.001 compared to WT $G\alpha_{i2}$ control. n=9–13 in each group. **b** Change in survival days after losartan treatment. Values are mean±standard error of the mean, n=9–13. ***p<0.001 for drug treatment compared to non-drug control. $^{†}p$<0.05 compared to respective $G\alpha_{i2}^{+/+}$ control. Differences were determined by one-way ANOVA with Bonferroni post-test

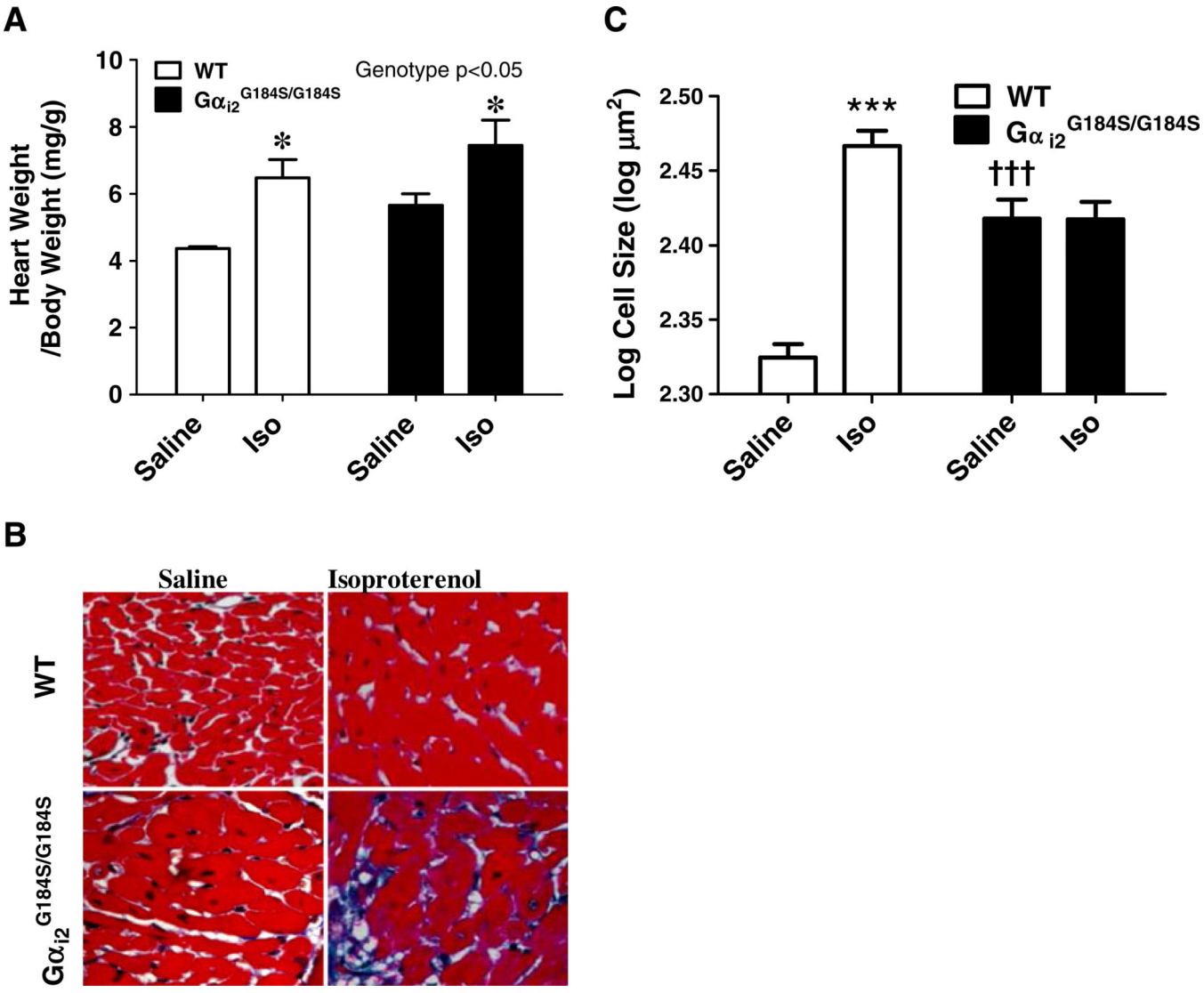

Fig. 4.

Iso-induced injury in WT and RGS-insensitive $G\alpha_{i2}^{G184S/G184S}$ mice. Mice were injected with saline or isoproterenol (30 mg/kg) daily for 7 days then tissues collected, weighed, and processed for microscopy as described in "Methods." **a** HW/BW after saline or Iso treatment in WT and RGS-insensitive $G\alpha_{i2}^{G184S/G184S}$ mice. **b** Images of transverse myocardial sections. **c** Myocyte cross sectional area measured using NIH-Image as described in "Methods." Values are mean±SEM, n =6–8 for HW/BW and >250 for myocyte size in each group. *p<0.05 or ***p<0.001 for Iso- vs saline-treated animals or $^{\dagger\dagger\dagger}p$<0.001 for WT vs $G\alpha_{i2}^{G184S/G184S}$by ANOVA

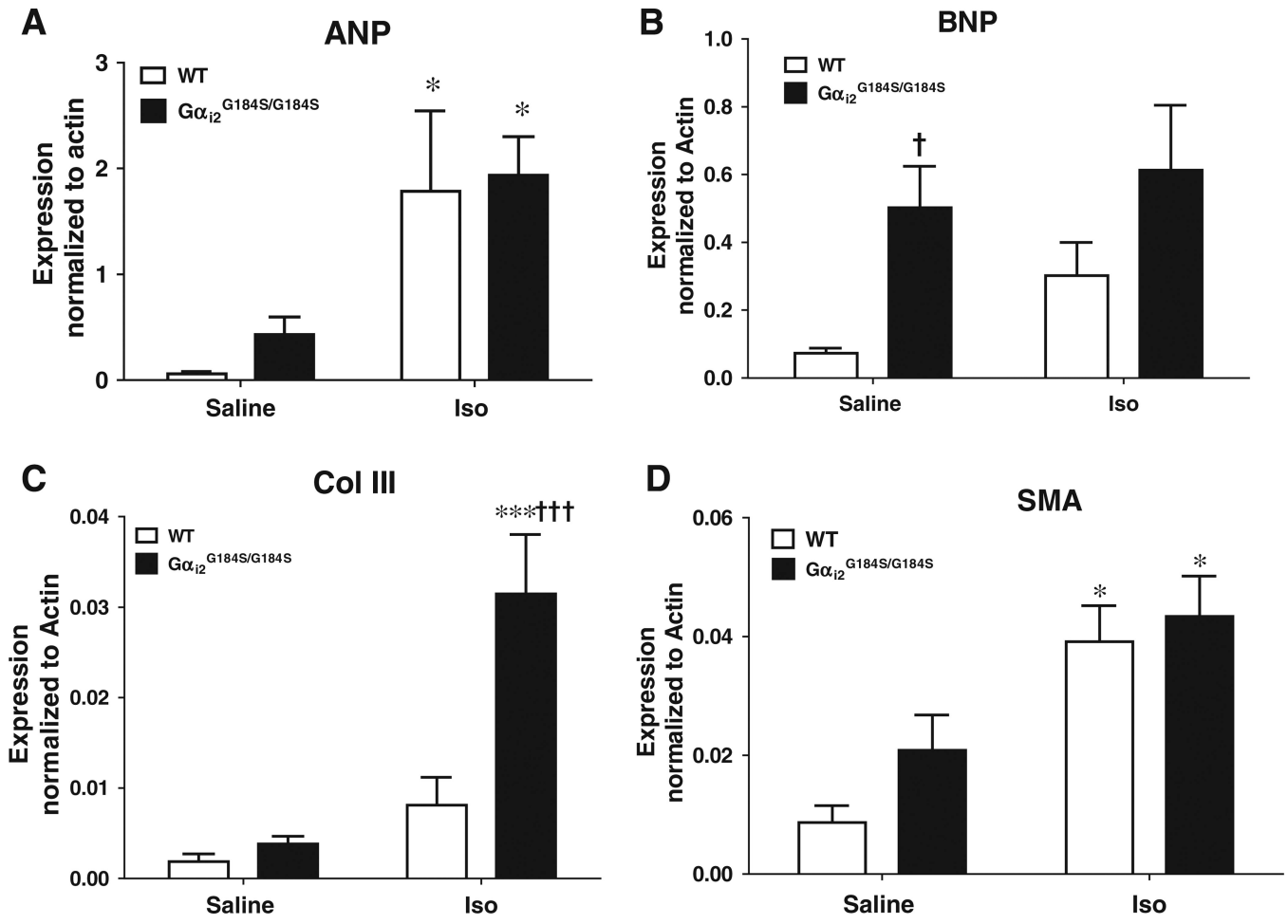

Fig. 5.

Expression of hypertrophy-associated genes. Real-time RT-PCR was performed on extracts of ventricular muscle from WT or $G\alpha_{i2}^{G184S/G184S}$ mice after 7 days of saline or Iso treatment. Expression was normalized to β-actin. **a** Atrial natriuretic peptide (ANP), **b** brain natriuretic peptide (BNP), **c** type III collagen (Col III), and d smooth muscle actin (SMA). Values are mean±SEM, n=6 in each group. *p<0.05 and ***p<0.001 Iso- vs saline-treated animals. $^{\dagger}p$<0.05 and $^{\dagger\dagger\dagger}p$<0.001, $G\alpha_{i2}^{G184S/G184S}$ vs WT animals

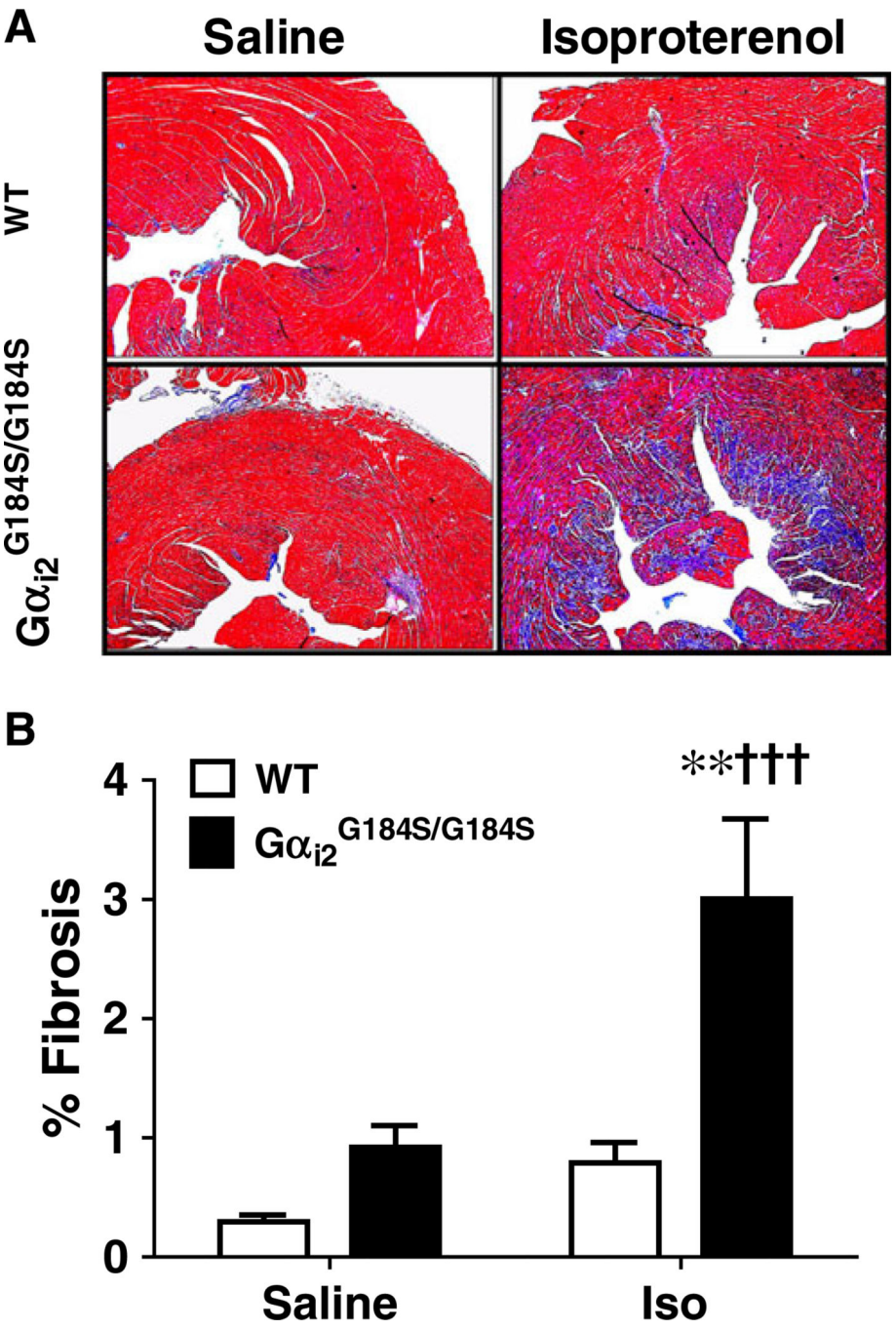

Fig. 6.

Increased fibrosis in $G\alpha_{i2}^{G184S/G184S}$ mutant hearts after Iso. **a** Cardiac sections were stained with Masson's trichrome after saline or isoproterenol treatment of wild-type (WT) or $G\alpha_{i2}^{G184S/G184S}$ mice. **b** Digital quantification of the tissue fibrosis was done as described in "Methods." Values are mean±SEM, n=6 in each group. **p<0.01 Iso- vs saline-treated animals. $^{\dagger\dagger\dagger}p$<0.001 $G\alpha_{i2}^{G184S/G184S}$ vs WT animals

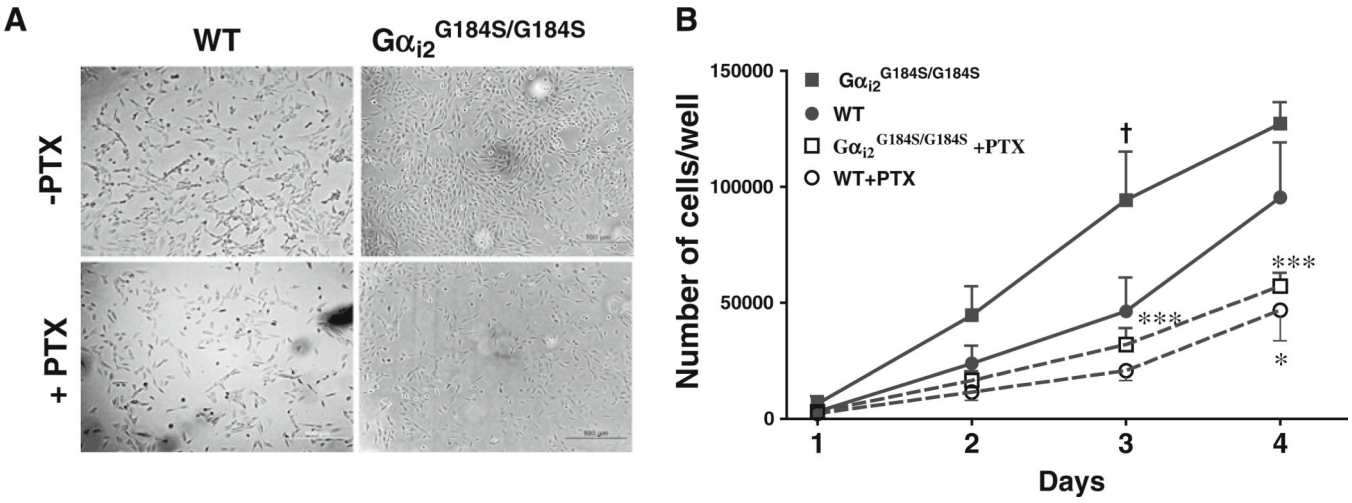

Fig. 7.

$G\alpha_{i2}$-dependent cardiac fibroblast proliferation. Cardiac fibro-blasts from wild-type (WT) and RGS-insensitive $G\alpha_{i2}^{G184S/G184S}$ hearts were grown for 3 days in 10% FBS without (PTX-) or with (PTX+) PTX. **a** DIC micrographs of cells. **b** Cell number was determined as described in "Methods." Values are mean±SEM for three different animals with each assay done in duplicate. $^{\dagger}p<0.05$ $G\alpha_{i2}^{G184S/G184S}$ vs WT cells $^{*}p<0.05$ or $^{***}p<0.001$ for the effect of PTX

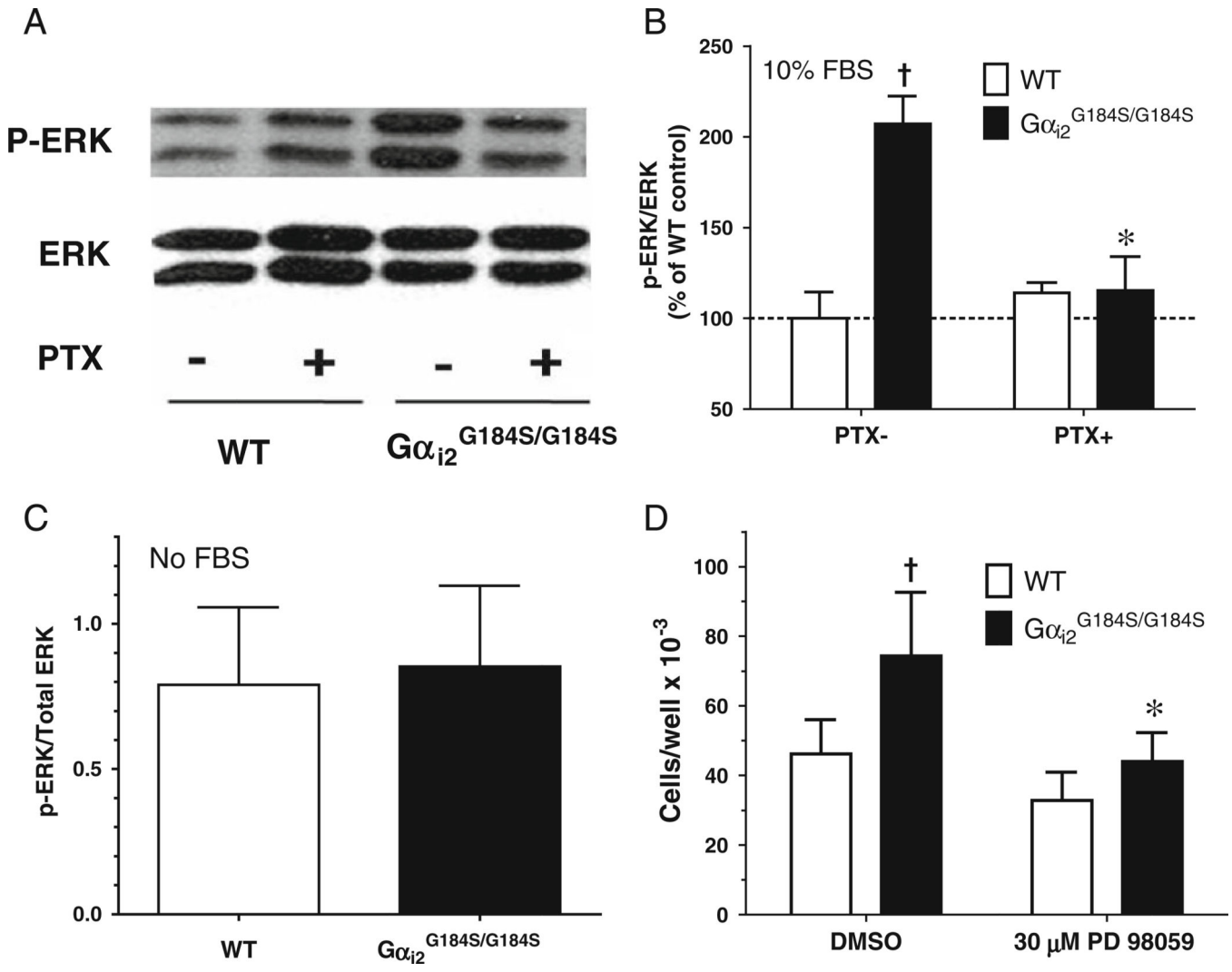

Fig. 8.

Increased ERK activity controls proliferation of RGS-insensitive $G\alpha_{i2}$ cardiac fibroblasts. Cardiac fibroblasts from wild-type (WT) or $G\alpha_{i2}^{G184S/G184S}$ mice were grown for 24 h in complete medium without (PTX-) or with (PTX+) PTX. **a** Representative blot for phospho-ERK and total ERK assessed as in "Methods." **b** Densitometric analysis of phospho-ERK/total ERK normalized to the value of the PTX-WT cells. **c** After growing for 24 h in complete medium, cardiac fibroblasts were starved with serum-free medium overnight, and samples were analyzed for phospho-ERK and total ERK expression. **d** The effect of the MEK inhibitor PD 98059 on fibroblast number was assessed after 3 days in culture with DMSO or 30 μM PD 98059. Values are mean±SEM for fibroblasts from three different animals with each assay done in duplicate. $^{\dagger}p<0.05$ $G\alpha_{i2}^{G184S/G184S}$ vs WT cells. $^{*}p<0.05$ vehicle- vs PTX- or PD 98059-treated cells

Table 1

Effect of RGS-insensitive $G\alpha_{i2}$ mutation ($G\alpha_{i2}^{G184S/+}$) on average age day of death in mice with cardiac-specific overexpression of Cre ($TG9^{+}$)

	$G\alpha_{i2}^{+/+}$	$G\alpha_{i2}^{G184S/+}$	$TG9^{+}$ $G\alpha_{i2}^{+/+}$	TG9+ $G\alpha_{i2}^{G184S/+}$
Male	ND	ND	86.7±1.3 (*n* = 11)	82.9±1.1* (*n*=9)
Female	ND	ND	81.2±0.5 (*n* = 13)	76.7±0.5** (*n*=12)

Values are mean±standard error of the mean, *n* represents animals in each group (9–13). Differences were determined by Mantel–Cox log rank test from the survival curve analysis

ND none died up to 95 days

* $p<0.05$

** $p<0.001$, indicate a significant effect on survival as compared to respective $G\alpha_{i2}^{+/+}$ control