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Activation of GPR30 inhibits cardiac fibroblast proliferation

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Abstract

The incidence of left ventricular diastolic dysfunction significantly increases in postmenopausal women suggesting the association between estrogen loss and diastolic dysfunction. The *in vivo* activation of G protein-coupled estrogen receptor (GPR30) attenuates the adverse effects of estrogen loss on cardiac fibrosis and diastolic dysfunction in mRen2.Lewis rats. This study was designed to address the effects of GPR30 on cardiac fibroblast proliferation in rats. The expression of GPR30 in cardiac fibroblasts isolated from adult Sprague-Dawley rats was confirmed by RT-PCR, Western blot analysis, and immunofluorescence staining. Results from BrdU incorporation assays, cell counting, carboxyfluorescein diacetate succinimidyl ester labeling in conjunction with flow cytometry and Ki-67 staining showed that treatment with G1, a specific agonist of GPR30, inhibited cardiac fibroblast proliferation in a dose-dependent manner, which was associated with decreases in CDK1 and cyclin B1 protein expression. In the GPR30-KO cells, BrdU incorporation, and CDK1 and cyclin B1 expression significantly increased when compared to GPR30-intact cells. G1 had no effect on BrdU incorporation, CDK1 and cyclin B1 mRNA levels in GPR30-KO cells. *In vivo* studies showed increases in CDK1 and cyclin B1 mRNA levels, Ki-67-positive cells, and the immunohistochemistry staining of vimentin, a fibroblast marker, in

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Declaration of Interests

There are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

the left ventricles from ovariectomized mRen2.Lewis rats versus hearts from ovary-intact littermates; two weeks of G1 treatment attenuated these adverse effects of estrogen loss. This study demonstrates that GPR30 is expressed in rat cardiac fibroblasts and activation of GPR30 limits proliferation of these cells likely via suppression of the cell cycle proteins, cyclin B1 and CDK1.

Keywords

estrogen; fibroblast; GPR30; heart; proliferation

Introduction

Left ventricular diastolic dysfunction (LVDD) is a very common asymptomatic condition in postmenopausal women which is associated with future diastolic heart failure (DHF) or HF with preserved ejection fraction [1–3]. LVDD is more prevalent in postmenopausal women than men or premenopausal women, in part due to the increased prevalence of hypertension and left ventricular hypertrophy that occurs after menopause [4–6]. Though estrogen loss is associated with the development of hypertension and LV hypertrophy, negative results in clinical trials of hormone replacement therapy have led to the speculation that estrogen replacement in the aging woman might not reverse cardiovascular damage or prevent further disease progression [7, 8]. There is increasing interest in developing alternative therapies for postmenopausal women that provide the benefits of estrogen replacement therapy, without its side effects and contraindications.

The G protein-coupled estrogen receptor (GPR30), also known as GPER, is a new estrogen receptor which was originally found in breast cancer cells located on the cell membrane and endoplasmic reticulum [9, 10]. It binds estrogen at high affinity and regulates proliferation of breast cancer cells, vascular smooth muscle cells and endothelial cells [11–19]. Subsequent studies have shown that GPR30 is widely distributed among a variety of mammalian tissues including the heart, lung, liver, adrenal gland, intestine, ovary, and brain, and is independent of species and sex [20, 21]. In the heart, GPR30 activation by its specific agonist, G1, improves contractile function and reduces infarct size in isolated rat and mouse hearts subjected to ischemia/reperfusion injury [22–25]. Our laboratory found that two weeks of subcutaneously administered G1 at the high dose of 400 μg/kg/day limited the adverse effects of a high salt diet or estrogen loss (by ovariectomy) on exacerbated increases in blood pressure in the female mRen2.Lewis rat, while the low dose (50 μg/kg/day) had no overt effects on blood pressure in this congenic strain. However, both doses of G1 attenuated the adverse effects of high salt or estrogen loss on cardiac fibrosis and diastolic function without other notable side effect [11, 26, 27]. These findings have expanded the mechanisms for estrogen-mediated cardioprotection by setting a new direction of research that focuses on the potential roles of GRP30 in the maintenance of cardiac structure and function in females after the cessation of ovarian hormone production.

Based on the findings that G1 limits interstitial collagen deposition associated with the loss of ovarian hormones in mRen2.Lewis rats and that GPR30 regulates cell proliferation in

breast cancer cells and cardiovascular cells [11–19], we hypothesized that that activation of GPR30 inhibits cardiac fibroblast (CF) proliferation leading to a reduction in cardiac collagen deposition, thereby attenuating cardiac remodeling and diastolic dysfunction. The *in vitro* and *in vivo* findings in this study support this concept in that GPR30 activation by G1 inhibited CF proliferation, in part, via its action on the regulation of CDK1 and cyclin B1 cell cycle pathways.

Materials and methods

Animal use

Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA) for cardiac fibroblast isolation. Female heterozygous mRen2.Lewis rats were obtained from the Hypertension and Vascular Research Center Congenic Colony at Wake Forest School of Medicine. All studies using animals were approved by the institution's Animal Care and Use Committee (A12-201), and all animal procedures conformed to the Guide to the Care and Use of Laboratory Animals published by the US National Institutes of Health. Rats were allowed to acclimate to controlled temperature (22 ± 2°C) and light (12 h light/dark cycles) with *ad libitum* access to food and water in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

Preparation and culture of adult rat cardiac fibroblasts

Cardiac fibroblasts (CFs) were isolated as described previously [28–31]. Briefly, the adult male Sprague-Dawley rats were deeply anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg) and then hearts were removed. Left ventricles (LVs) were minced and digested with collagenase type II (100 U/mL, Invitrogen, Carlsbad, CA, USA) and 0.1% trypsin (Invitrogen, Carlsbad, CA, USA), and liberated cells were collected by centrifugation at 400 *g* for 10 minutes. Pelleted cells were resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL). Cells were seeded onto a 75-cm^2 flask and incubated in a humidified 5% CO_2/95% air atmosphere at 37°C, and after 3 h unattached cells were removed by changing the culture medium, and attached cells were cultured further for 2 days. According to the literature, the purity of cultured CFs was greater than 98% using this method of isolation [28–31]. This study also demonstrated that all the cultured CFs were positively stained with a CF marker, vimentin (Fig. 1C).

Cardiac fibroblasts were used for experiments at passages 4–6. Before each experiment, cells were seeded in six-well plates or chamber slides (Lab-Tek™ Chamber Slides: Thermo Scientific NUNC 177437) at the density of 3×10^4 cells/cm^2 and starved for 18 h in DMEM containing 0.1% FBS. Cells were subsequently treated with G1 (10^{-10}–10^{-5}M, Cayman Chemical Company) for 24-h, and were then fixed with cold methanol for immunocytochemical staining, lysed with TRIzol (Invitrogen, Carlsbad, CA, USA) for real-time PCR analysis, or trypsinized for protein analysis.

Immunocytochemistry staining in cardiac fibroblasts

Following treatment, the cells cultured on chamber slides were fixed with ice-cold 100% methanol for 10 min, and incubated with primary antibody against vimentin (1:1000, IgG Corp, Nashville, TN, USA), GPR30 (1:1000, Bioss. Woburn, MA, USA), or Ki-67 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Cells were washed with PBS and then incubated with secondary IgG antibodies conjugated to Alexa 488 or 555 (1:500, Invitrogen, Carlsbad, CA, USA) for 45 min at room temperature. The nuclei were counterstained with TP-PRO-3. Excess antibody was removed by three washes and cells were imaged with a Zeiss LSM-510 confocal microscope.

Bromodeoxyuridine (BrdU) incorporation assay

Cell proliferation was measured using the BrdU Cell Proliferation Assay Kit (Millipore #2750, Billerica, MA, USA). Cardiac fibroblasts seeded at 2×10^3 per well in a 96-well cell culture plate were serum-starved for 18 h, and then changed to medium with 10% FBS and treated with G1 at various doses (10^{-10}–10^{-5}M) for 2 h. 20 μl of BrdU labeling solution was subsequently added to each well and cells were incubated for an additional 18 h. BrdU incorporation assay was performed immunochemically using an anti-BrdU monoclonal antibody followed by incubation with peroxidase-conjugated second antibody and TMB peroxidase substrate according to the manufacturer's instructions. The absorbance of the assay wells was measured using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA) at wavelengths of 450 nm.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and flow cytometry analysis

For CFSE staining, cells were harvested and washed two times with PBS, then stained with 5 μM CFSE (Invitrogen, Carlsbad, CA, USA) for 10 min at room temperature. Residual CFSE was removed by washing twice with PBS and cells were seeded in 6-well plates and grown in cell culture medium. The labeled cells were cultured in the presence or absence of G1 for 3 or 7 days and the CFSE fluorescence intensity was analyzed by BD FACSCalibur analyzer (BD Biosciences, San Jose, CA) and analyzed using BD CellQuest software. With cell division and proliferation, the CFSE staining is expected to be diluted and the fluorescence strength shifted to the left (closer to the control cells).

GPR30 knockdown in CFs with small interfering RNA (siRNA)

GPR30 siRNA and scrambled siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CFs were seeded in 6-well plates at a density of 1×10^5 cells per well with DMEM containing 10% FBS without antibiotics until 50–70% of confluence. siRNA (20 nM) against GPR30 or scrambled siRNA were transfected into cells using Lipofectamine® RNAiMAX Reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions after cells were starved for 24 h in Opti-MEM® I Reduced Serum Medium (Life Technologies, Grand Island, NY). Transfected cells were used for the experiments after 48 hours of siRNA transfection.

In vivo experimental protocol

The OVX-mRen2.Lewis rat is a well-established animal model that emulates the cardiovascular phenotype of the postmenopausal woman [11, 26, 32]. At 4 weeks of age, female mRen2.Lewis rats were randomly assigned to undergo either OVX (n=16) or sham operation (Sham; n=10) performed, as previously described [11, 26]. Once the rats reached 13 weeks of age, the OVX group was randomly divided to receive either the GPR30 agonist, G1, (OVX-G1; n=7; Cayman Chemical Company, Ann Arbor, MI, USA) diluted in a DMSO/EtOH mixture for a targeted dose of 50 mcg/kg/day or DMSO/EtOH vehicle (OVX-V; n=9) administered subcutaneously via an osmotic mini-pump (DURECT Corporation, Cupertino, CA, USA) for 2 weeks. This dose of G1 was determined by an initial pilot study conducted in our laboratory that demonstrated lusitropic effectiveness without overt reductions in systolic blood pressure [27]. Rats were euthanized via exsanguination by cardiac puncture while under deep anesthesia (pentobarbital 50 mg/kg) at 15 weeks of age. The LV was isolated and cut into pieces for biochemical and histological analyses.

Analysis of gene expression by quantitative real-time PCR

Real-time PCR was used to detect gene mRNA levels in cells and cardiac tissues, as previously described [11]. Total RNA was extracted from cultured CFs and from frozen, pulverized LV tissue of mRen2.Lewis rats using TRIzol Reagent (Qiagen Inc, CA). Complementary first strand DNA was synthesized using the Omniscript RT kit (Qiagen Inc, CA). Relative quantification of mRNA levels by real-time PCR was performed using a SYBR Green PCR kit (Qiagen Inc, CA). Amplification and detection were performed with the ABI7500 Sequence Detection System (Applied Biosystems). Sequence-specific oligonucleotide primers were designed according to published GenBank sequences (www.ncbi.nlm.nih.gov/Genbank) and confirmed with OligoAnalyzer 3.0. The relative target mRNA levels in each sample were normalized to S16 ribosomal RNA. Expression levels are reported relative to the geometric mean of the control group.

Western blot analysis

Cultured CFs or LV tissue homogenates from mRen2.Lewis rats were separated by SDS–PAGE and immunoblots were probed using antibodies for GPR30 (1:500, Bioss. Woburn, MA, USA), CDK1 (1μg/ml, Abcam, Cambridge, MA, USA), and cyclin B1 (1μg/ml, Abcam, Cambridge, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5,000; Cell Signaling, Danvers, MA, USA) was used as a loading control. The bands were digitized using MCID image analysis software (Imaging Research, Inc., Ontario, Canada). Each band was expressed in arbitrary units and normalized to its own GAPDH.

Immunohistochemistry staining of vimentin in heart tissues

Immunohistochemistry staining of formalin-fixed and paraffin-embedded heart sections was performed using standard procedures. LV sections were incubated with antibodies against vimentin (1:1000; Abcam, Inc., Cambridge, MA, USA), or Ki-67 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, rinsed with phosphate-buffered saline, and incubated with biotinylated secondary IgG (Vector Laboratories, Burlingame, CA) for 3 h at 4°C. Antibody binding was detected with the Vectastain ABC Elite avidin/

biotin/peroxidase kit (Vector Laboratories, Burlingame, CA, USA). The tissue sections were observed under light microscopy. Pictures were taken from ten random fields and NIH ImageJ software was used to quantify the staining in the heart.

Statistical analysis

All results are reported as mean ± SEM. For all endpoints, one-way ANOVA was used to determine the significance of differences among groups. Significance of interactions between groups was determined using *Tukey* post-*hoc* tests. Differences for all tests were considered significant at $P<0.05$. Analyses were performed using GraphPad Prism, version 6 (GraphPad, San Diego, CA, USA).

Results

GPR30 expression in cultured cardiac fibroblasts

To study the effects of GPR30 on CF proliferation, we first determined the expression of GPR30 in these cells. Results from RT-PCR show that the GPR30 gene exists in CFs isolated from adult male Sprague-Dawley rats (Fig. 1A). Furthermore, using a specific antibody to GPR30, we found a single band in Western blot analysis corresponding to the appropriate molecular weight of GPR30 (~50 kDa) (Fig. 1B). Using this antibody, immunofluorescence staining with confocal microscopy further confirmed GPR30 protein expression in cultured CFs (Fig. 1C).

G1 inhibited cardiac fibroblasts proliferation *in vitro*

In the cultured CFs isolated from adult male Sprague-Dawley rats, treatment with G1 significantly inhibited cell growth (Fig. 2A). Quantification data showed that cell number was decreased by 14% after G1 treatment at the concentration of 10 μM for 48 h (Fig. 2B). Using a more sensitive method for cell proliferation, results from the BrdU incorporation assay show that G1 treatment for 24 h significantly inhibited BrdU incorporation in CFs at concentrations starting from 0.01 μM, with a maximum inhibition of 70% at the concentration of 10 μM, compared with vehicle treated cells (Fig. 2C).

To corroborate the above findings, we employed the carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and flow cytometric assay to test if G1 inhibits proliferation of cultured CFs. Cardiac fibroblasts were labeled with CFSE and cultured in the presence or absence of G1 at various concentrations for 3 or 7 days and analyzed by flow cytometry. With the cell division and proliferation, it's expected that the CFSE staining will be diluted and the fluorescence strength shifted to the left (closer to the control cells). In Fig. 3A, the black color represents control cells without CFSE labeling, showing the background of fluorescence, and the red color represents vehicle-treated cells. We found a dose-dependent increase in fluorescence strength in the G1 treated cells, suggesting that the CFSE labeling was not as diluted in these cells, when compared to vehicle-treated cells (Fig. 3A). Therefore, results from CFSE labeling and the flow cytometric assay reveal that G1 inhibits cell division and proliferation in CFs.

Ki-67 is a cellular marker for proliferation and is strictly associated with cell proliferation. It is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). Immunofluorescence staining with Ki-67 antibody showed the intensity of Ki-67 staining in G1 treated cells was also weaker when compared to vehicle-treated cells (Fig. 3B).

G1 inhibited CDK1 and cyclin B1 expression in cultured cardiac fibroblasts

To further determine the mechanisms of CF proliferation inhibition by G1, we measured cell cycle related gene mRNA levels in CFs treated with various concentrations of G1 for 24 h using real-time PCR. Fig. 4A and 4B show that G1 decreased CDK1 and cyclin B1 mRNA levels in a dose-dependent manner, with the maximum inhibition of ~70% for both CDK1 and cyclin B1 at the concentration of 10 μM of G1 treatment. Western blot analyses further confirmed these results as G1 treatment decreased both CDK1 and cyclin B1 protein levels in CFs (Fig. 4C–E).

G1 treatment at the concentrations of 0.01–10 μM for 24 h did not change the mRNA levels of cdc25c, cyclin A2, cyclin D1, and cyclin D2 in CFs determined by real-time PCR (Fig. S1). Cyclin D3 mRNA levels tended to decrease in G1 treated cells compared to vehicle but the data did not reach statistical significance (Fig. S2A).

GPR30 knockdown prevented the effects of G1 in cardiac fibroblasts

To more specifically determine the effects of GPR30 on CF proliferation, we knocked down GPR30 in cultured CFs using siRNA specific to GPR30. Fig. 5A shows GPR30 mRNA levels in CFs at 48 h after siRNA transfection. GPR30 siRNA decreased GPR30 mRNA by 80% compared to control siRNA (Fig. 5A). The GPR30 knockdown was also confirmed with western blot analysis using a specific antibody to GPR30 (Fig. 5B). Interestingly, in the vehicle-treated cells, BrdU incorporation increased by 3.1-fold in GPR30-KO vs. GPR30-intact cells ($P<0.01$). As expected, 1 μM of G1 treatment for 24 h decreased BrdU incorporation in GPR30-intact cells (Fig. 5C) compared with vehicle -treated cells ($P<0.01$), while G1 treatment had no effect on GPR30-KO cells (Fig. 5C).

Correspondingly, both mRNA and protein levels of CDK1 and cyclin B1 were significantly increased in GPR30-KO compared with GPR30-intact cells (Fig. 5D–F), and G1 treatment lessened the expression of these genes in GPR30-intact cells ($P<0.05$). Importantly, these changes were absent in GPR30-KO cells (Fig. 5D and E).

Effects of GPR30 on collagen related gene expression in cardiac fibroblasts

To determine if the activation of GPR30 affects collagen production from CFs, COL1A1, COL1A2, and COL3A1 mRNA levels were determined by real-time PCR in CFs treated with various concentrations of G1. Treatment of G1 for 24 h decreased COL1A1 mRNA by 20% in CFs at the concentration of 10 μM, while no effect was observed at the concentrations of 0.01–1 μM (Fig. 6A). G1 did not change COL1A2 mRNA levels in CFs, but it tended to decrease COL3A1 mRNA at the high concentration of 10 μM (Fig. 6A).

Matrix metalloproteinases (MMPs) have a major role in cardiac collagen deposition and heart remodeling, via actions on the proteolytic degradation of extracellular collagens, and a number of bioactive molecules [33–35]. In this study, G1 treatment for 24 h at the concentrations of 0.01–10 μM did not change mRNA levels of MMP-2, MMP-3, and MMP-9 in CFs (Fig. 6B). However, G1 increased MMP-12 mRNA levels in CFs in a dose-dependent manner, with a 4-fold increase in MMP-12 gene expression at the concentration of 10 μM of G1 compared with vehicle treated cells. Interestingly, G1 also inhibited mRNA expression of the endogenous tissue inhibitor of metalloproteinase (TIMP)-1, while it did not affect mRNA levels of TIMP-4 and TGF-β1 (Fig. 6B).

In GPR30-KO CFs, mRNA levels of COL1A1, COL1A2, and COL3A1 increased by 1.9–2.8 folds compared with GPR30-intact cells ($P<0.01$). Consistent with the results from G1 treated cells, MMP-12 mRNA decreased by 78%, and TIMP-1 increased by 2.2 folds in GPR30-KO vs. GPR30-intact cells ($P<0.01$) (Fig. 6C).

Gene expression of the molecular markers for cardiac fibroblast during *in vitro* experiments

Since fibroblasts might differentiate to myofibroblasts in culture with serum, we studied the changes in gene expression of the molecular markers for fibroblasts and myofibroblasts, including fibronectin, discoidin domain receptor (DDR) 2, and most importantly, α smooth muscle actin (αSMA), which is highly expressed in myofibroblasts, but not in fibroblasts [36]. The results showed that various concentrations of G1 treatment for 24 h did not affect either fibronectin or DDR2 mRNA levels in CFs. Interestingly, G1, at the concentration of 10 μM, significantly decreased αSMA mRNA by 77% in CFs ($P<0.05$), while there was only a tendency to decrease gene expression of this myofibroblast biomarker at the concentrations of 0.01–1 μM ($P>0.05$) (Fig. S3).

In GPR30-KO vs. GPR30-intact cells, fibronectin mRNA significantly increased by 2.3 fold ($P<0.01$), while DDR2 and αSMA mRNA levels tended to increase but did not reach statistical significance (Fig. S3).

G1 inhibited cardiac fibroblast proliferation *in vivo*

To study GPR30 and cardiac fibroblast proliferation *in vivo*, we used the OVX female mRen2.Lewis rat, an estrogen-sensitive model that emulates the cardiovascular phenotype of the postmenopausal woman [27]. In the present study, we found a significant increase in the immunohistochemical staining of vimentin, a fibroblast marker, in hearts from OVX mRen2.Lewis rats when compared to hearts from sham-operated controls ($P<0.05$). The increased expression of vimentin following estrogen loss was significantly inhibited by G1 treatment for two weeks (Fig. 7). Thus, estrogen loss in the female mRen2.Lewis rat was associated with an increase in CFs, and this was counteracted by G1 treatment.

To study if CDK1 and cyclin B1 genes are also involved in CFs proliferation *in vivo*, we determined CDK1 and cyclin B1 mRNA levels in the LVs of mRen2.Lewis rats using real-time PCR. CDK1 and cyclin B1 mRNA levels increased in OVX rats by 60% and 51%, respectively, compared to sham-operated rats ($P<0.05$), while these increases were

significantly inhibited by G1 treatment (Fig. 8A and B). Immunohistochemical staining of Ki-67, a proliferation marker, also showed that there were more Ki-67 positive cells in the hearts of OVX rats than in hearts from sham-control rats. G1 treatment blocked the increase of Ki-67 positive cells in the hearts of OVX rats (Fig. 8C).

Discussion

GPR30 is a seven transmembrane domain protein identified as a novel estrogen binding protein structurally distinct from the classic estrogen receptors α and β (ERα and ERβ). Its agonist, G1, has been generated with high selectivity and affinity to GPR30, and little to no affinity for ERα and ERβ [37]. G1 has no overt binding affinity to 25 other G protein-coupled receptors [38]. Using G1, our results from *in vitro* and *in vivo* studies strongly suggest that inhibition of CF growth is attributable to activation of GPR30. This premise was further confirmed by *in vitro* experiments showing that the knockdown of GPR30 in CFs by siRNA effectively blocked the inhibitory action of G1 on CF growth and proliferation. In support of these findings, we further showed that GPR30 is expressed in CFs from adult rats.

In vivo and *ex vivo* studies have previously demonstrated that GPR30 activation by G1 improves heart function and limits remodeling in both female and male rodent models of ischemia/reperfusion injury [22, 25], suggesting that GPR30 is expressed in the heart of both sexes. To date, there is no study that identifies the distribution of GPR30 within the cells of the heart, i.e., cardiomyocytes and non-cardiomyocytes. While chronic G1 treatment attenuated cardiac fibrosis and cardiomyocyte hypertrophy in our previous study [11, 26], we cannot assume that GPR30 is in fact expressed in both cardiomyocytes and fibroblasts without further evidence. To our knowledge, this is the first report that clearly demonstrates GPR30 expression in CFs derived from rats. Although the activation of GPR30 might directly affect cardiomyocytes [11], cardiac vascular smooth muscle cells, and endothelial cells [38, 39], the presence of GPR30 in CFs suggests that the protective effects of estrogen or G1 on cardiac fibrosis and remodeling might be, in part, due to the activation of GPR30 in CFs.

The direct action of GPR30 on CFs and the cardiac extracellular matrix may underlie its anti-remodeling effect and the attenuation of diastolic dysfunction following estrogen loss [11]. 17β-estradiol (E2) has been previously shown to regulate the proliferation of CFs and the capacity of these cells to produce collagen [29, 31, 40]. Our recent work shows that the GPR30 agonist G1 limits cardiac interstitial collagen deposition associated with high salt diet or the loss of ovarian hormones in mRen2.Lewis rats [11, 26]. The present study extends these findings in that G1 directly inhibited proliferation of CFs cultured in medium with FBS and decreased mRNA levels of cell cycle-related genes, CDK1 and cyclin B1. Hearts rendered from OVX mRen2.Lewis rats further confirm these findings by showing increased LV CF number, CF proliferation, and CDK1 and cyclin B1 gene expression, when compared to LVs from sham-operated controls, while chronic G1 treatment for 2 weeks limited these increases. Considering that the dose of G1 used in the *in vivo* study did not change blood pressure [27], taken together with the presence of GPR30 in CFs and the *in vitro* findings, the inhibitory effect of G1 on CF proliferation *in vivo* is most likely due to its

direct action rather than other indirect or systemic effects on the heart. Besides immunohistochemical staining marking fibroblast presence, the staining for a proliferation marker, Ki-67, was also employed in the *in vivo* study. Because the proliferation of cardiomyocytes is very rare and the non-cardiomyocytes account for approximately 70% of the total cell number in the heart, with the vast majority being fibroblasts, the results from *in vivo* Ki-67 staining most likely reflect the proliferation of CFs.

Although, until now, there have been no reports of GPR30 and cell proliferation in the heart, GPR30 has been shown to regulate cell proliferation in other cell types. Consistent with the present study, Teng et al. showed that normal and malignant bladder urothelial cells respond to GPR30 activation by reducing cell proliferation [12]. More recent studies have also shown that the activation of GPR30 by G1 leads to growth inhibition of prostate cancer cells both *in vitro* and *in vivo* [13]; however, opposite results have been seen in different cell types. GPR30 mediates the proliferative effects of estradiol in thyroid [14], endometrial [14, 15], ovarian [16], and breast cancer cells [16, 17]. Taken together with our findings, GPR30 in the regulation of cell growth may depend on the cell type and/or pathologic condition.

GPR30 may affect cell proliferation through its regulation of cycle-related genes. In human urothelial cells, GPR30 overexpression abolished E2-induced c-fos, c-jun, and cyclin D1 expression, while inactivation of GPR30 by small interfering RNA increased these gene expressions, suggesting that GPR30-mediated inhibition of urothelial cell proliferation is the result of a decrease in cyclin D1 [12]. However, in human thyroid carcinoma cells and BG1 ovarian cancer cells, G1 promotes cell proliferation associated with an up-regulation of cyclin A1, cyclin E, and cyclin D1 [14, 16]. In this study, the inhibition of GPR30 on CF proliferation associated with decreases in cycle-related genes CDK1 and cyclin B1. G1 did not affect mRNA levels of cyclin D1, cyclin D2, cyclin A2, and cdc25c in CFs in the presence of serum. It appears that the effects of GPR30 on cell cycle-related genes differ depending on the cell type, which, in turn, leads to distinct effects on cell growth.

Cardiac fibroblasts are the main source of collagen production in the heart, which is the primary component of the extracellular matrix in heart tissue [41]. Collagen type I is the major collagenous product of CF, representing 80% of the total newly synthesized collagens [42]. Actually, changes in collagen type, rather than the total collagen content, have an important role in determining the structural and functional properties of the heart [43]. Increased collagen type I/III ratio is associated with an increase in LV mass and the onset of cardiac fibrosis after ovariectomy in aged rat hearts [44]. In our study, 10 μM of G1 decreased COL1A1 mRNA, but did not significantly affect COL3A1 mRNA expression in cultured CFs. While our study focused on CF proliferation, rather than collagen production from fibroblasts, the findings by Xu et al. [44] provide reason for future studies to focus on the effects of GPR30 on collagen production and the proportions of the various collagen types within the heart and the significance these GPR30-related effects may have on cardiac remodeling.

Besides collagen production from CFs, MMPs and TIMPs also influence LV structural and functional properties by governing ECM turnover and remodeling [33–35]. Abnormalities in MMP and TIMP expression have been described in various cardiovascular diseases [33–35].

However, the influence of estrogen on the MMP and TIMP balance in cardiovascular diseases has not been fully studied. G1 treatment increased MMP-12 mRNA and inhibited mRNA expression of TIMP-1 in CFs in a dose-dependent manner. While the mechanisms and significance of the regulation of MMP/TIMP by GPR30 is not clear from the present data, our results suggest GPR30 in the heart might affect cardiac collagen deposition through multiple pathways involving CF proliferation, collagen production from CFs, and MMP/TIMP balance.

Limitations and future studies

It has been reported that the cultured adult cardiac fibroblasts only retain fibroblast characteristics in early passage cultures, as they gradually transdifferentiate into myofibroblasts. The first myofibroblast markers appear as early as the first passage, when compared with that in quiescent P0 fibroblasts [36]. While other CF proliferation studies used cells in culture following more than three passages, as we did in the present study, it would be greatly beneficial to compare the effects of GPR30 on CF proliferation and collagen production in cells cultured at various passages. Interestingly, the mRNA level of αSMA, a molecular marker used to distinguish myofibroblast from fibroblast, significantly decreased after 24 hours of high-dose G1 (10 μM), while there was only a tendency for decreased expression at the lower doses of G1. Moreover, αSMA mRNA levels tended to increase in GPR30-KO vs. GPR30-intact cells. These results raise new and interesting questions about the potential role of GPR30 in the de-differentiation of myofibroblasts to fibroblasts, which deserve further investigation [45].

Another limitation is that in our *in vitro* studies, male adult CFs were used and were not compared with female cells. It was reported that the activation of GPR30 by G1 protects the heart from ischemia/reperfusion injury in a gender-independent manner [22, 25]. However, interestingly, male, but not female, GPR30-deficient mice suffer from impaired LV structure and function with enlargement of the LV and reductions in LV contractility and relaxation capacity [46]. It is clear that GPR30 has important cardioprotective roles in both male and female rodents. However, the responses and behavior of CFs to the activation of GPR30 might be different considering the sex-related differences in the proliferative response of CFs to hypoxia and estrogen [47]. Comparing the responses of CFs to GPR30 and the different signaling pathways that may be involved in male and female CFs remains to be studied.

Only one dose of G1 (50 μ/kg/day) was used in the *in vivo* study. Although chronic treatment with this dose of G1 has been demonstrated to have lusitropic effectiveness without overtly affecting blood pressure in OVX mRen2.Lewis rats [27], it might be important to investigate the effects of different doses of G1 on cardiac fibroblast number in future *in vivo* studies.

Clinical implications

Heart disease is the leading cause of death in women over 40-years of age and the risk of heart disease in women increases after menopause. LVDD is a common cardiac abnormality in postmenopausal women that may progress to DHF [24]. So far there are no proven

pharmacologic therapies that delay or reverse age-related LVDD or subsequent DHF [48]. To discover new targets and develop alternative therapies for LVDD and DHF that achieve the cardiovascular benefits of estrogen replacement therapy without its side effects and contraindications [49, 50], we need a better understanding of the role of estrogen and its receptors, in both cardiomyocytes and fibroblasts, and the downstream signaling pathways involved. This study provides evidence and mechanisms for the protective roles of GPR30 in limiting cardiac fibrosis; which is a widely found factor that contributes to the development of LVDD and DHF in postmenopausal women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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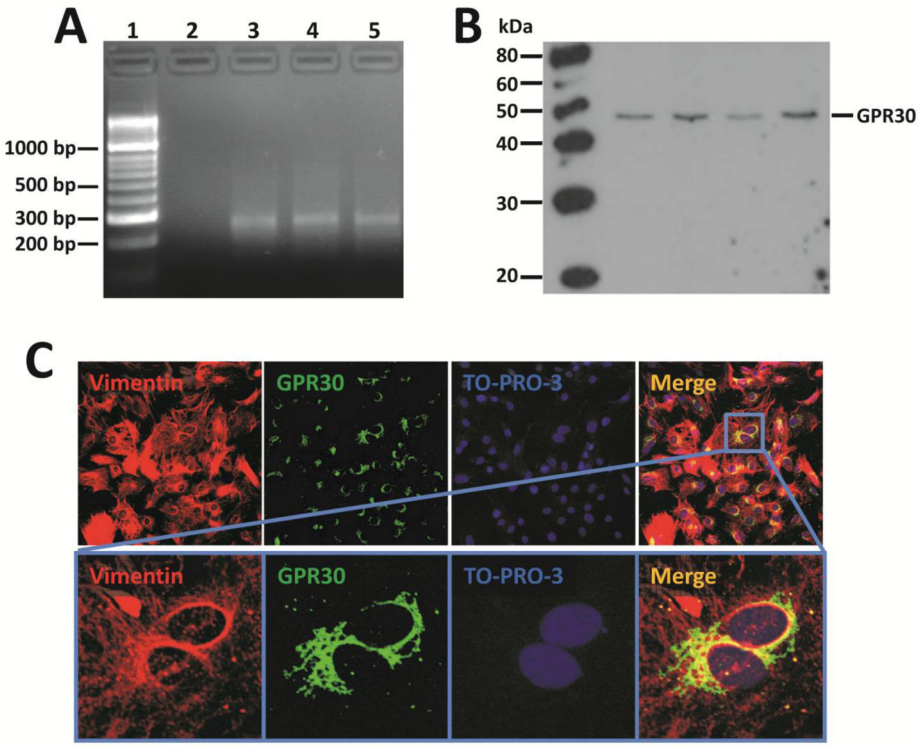


Fig. 1. GPR30 expression in cultured rat cardiac fibroblasts

(A) RT-PCR amplification of a 275-bp GPR30 fragment in cardiac fibroblasts. Lane 1: marker; lane2: negative control (without cDNA); lane 3–5: cardiac fibroblasts. (B) Western blot of cardiac fibroblasts showing a single band corresponding to the appropriate molecular weight of GPR30 (~50 kDa). (C) Confocal microscopy images of immunofluorescence staining for vimentin (red), GPR30 (green), and TO-PRO-3 (blue) in cultured cardiac fibroblasts. Lower panels are magnified to show the specific location of GPR30 expression in cardiac fibroblasts.

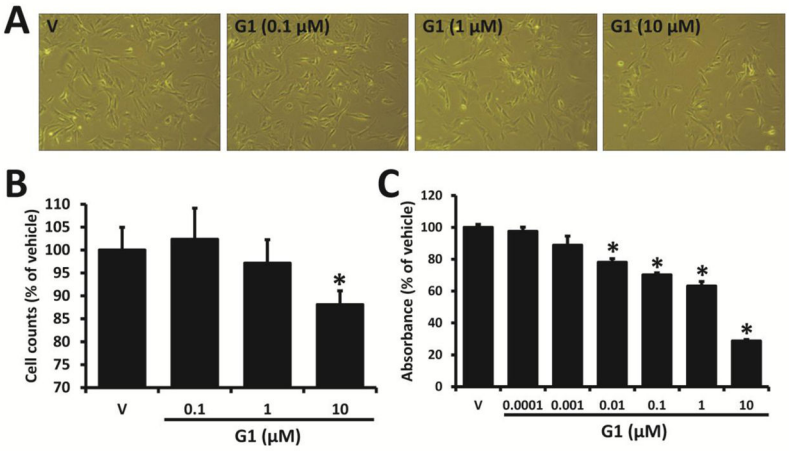


Fig. 2. G1 inhibits cardiac fibroblast growth and BrdU incorporation

(A) Representative images of four independent experiments showing G1 treatment for 24 hours inhibits cardiac fibroblast growth in a dose-dependent manner. (B) Cell number after 24 hours of G1 treatment (n=4). (C) G1 inhibits BrdU incorporation in cultured cardiac fibroblasts (n=4). Values are mean ± SEM; * P<0.05 vs. vehicle.

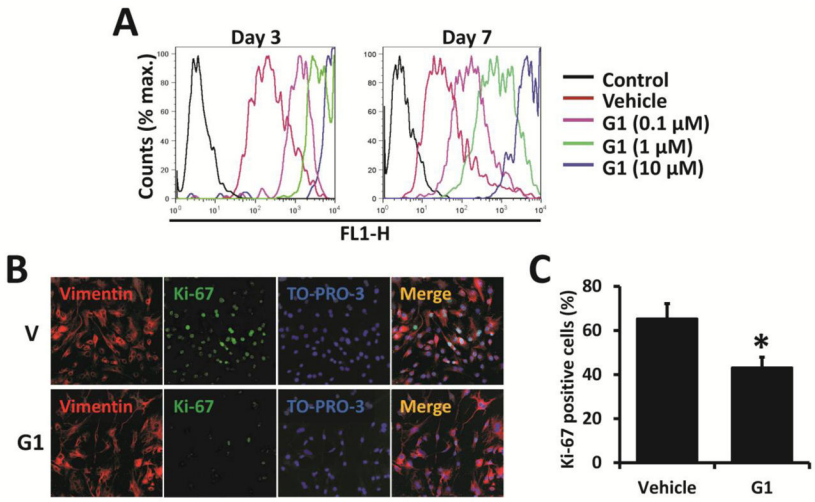


Fig. 3. Flow cytometry analysis of cell division and Ki-67 expression

(A) Flow cytometry analysis of cell division at day 3 and day 7 after carboxyfluorescein succinimidyl ester (CFSE) labeling in cardiac fibroblasts with various doses of G1 treatments. (B) Confocal microscopy images of immunofluorescence staining for vimentin (red), Ki-67 (green), and TO-PRO-3 (blue) in cultured cardiac fibroblasts treated with vehicle (upper panel) or 1 μM of G1 (lower panel). (C) Quantification of Ki-67 staining showing the percentage of Ki-67 positive cells over total cells. Values are mean ± SEM; n=3; * $P<0.05$ vs. vehicle.

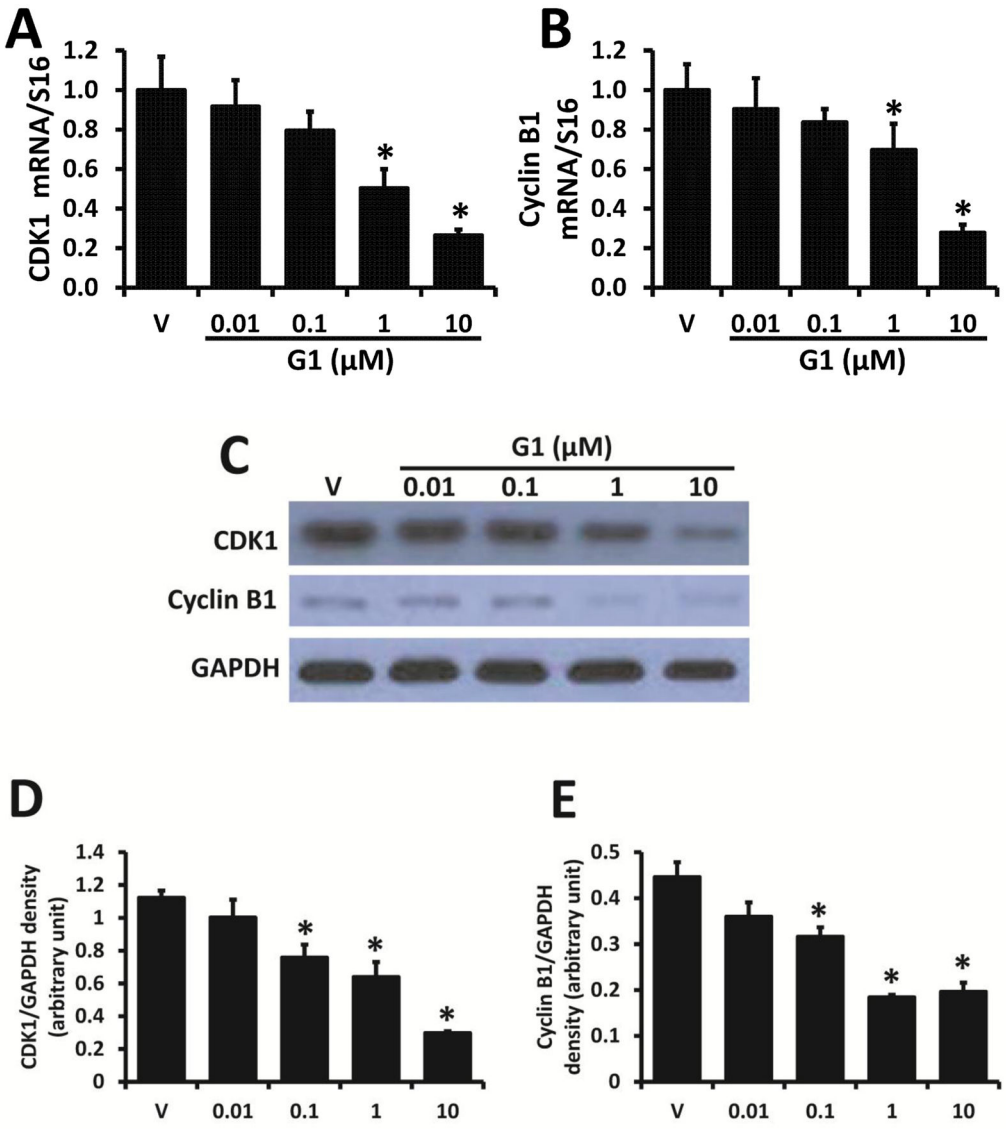
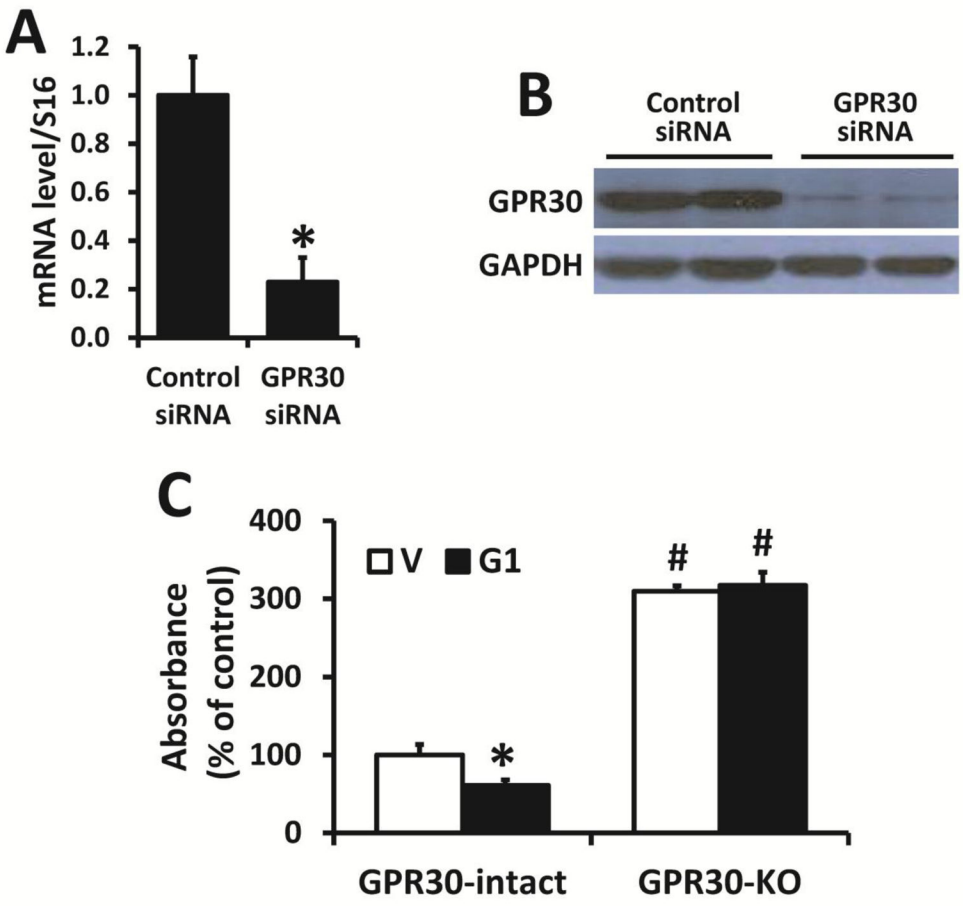


Fig. 4. G1 inhibits CDK1 and cyclin B1 expression in cardiac fibroblasts

(A) CDK1 and (B) cyclin B1 mRNA levels determined by real-time PCR in cardiac fibroblasts treated with various doses of G1. Values are mean ± SEM; n=4; * $P<0.05$ vs. vehicle. (C–E) Representative images and quantification of three independent experiments of Western blot analysis showing G1 inhibits protein levels of CDK1 and cyclin B1 in a dose-dependent manner. Values are mean ± SEM; * $P<0.05$ vs. vehicle.



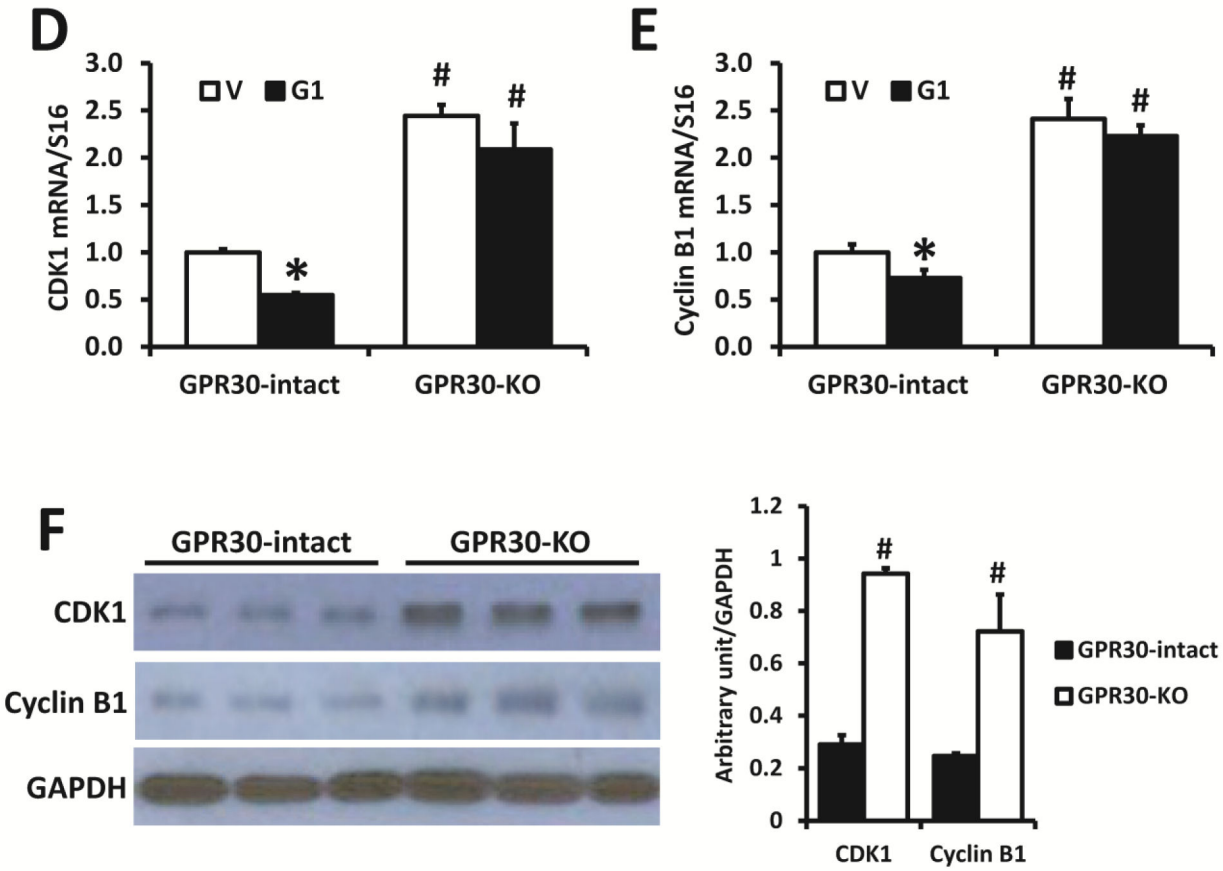
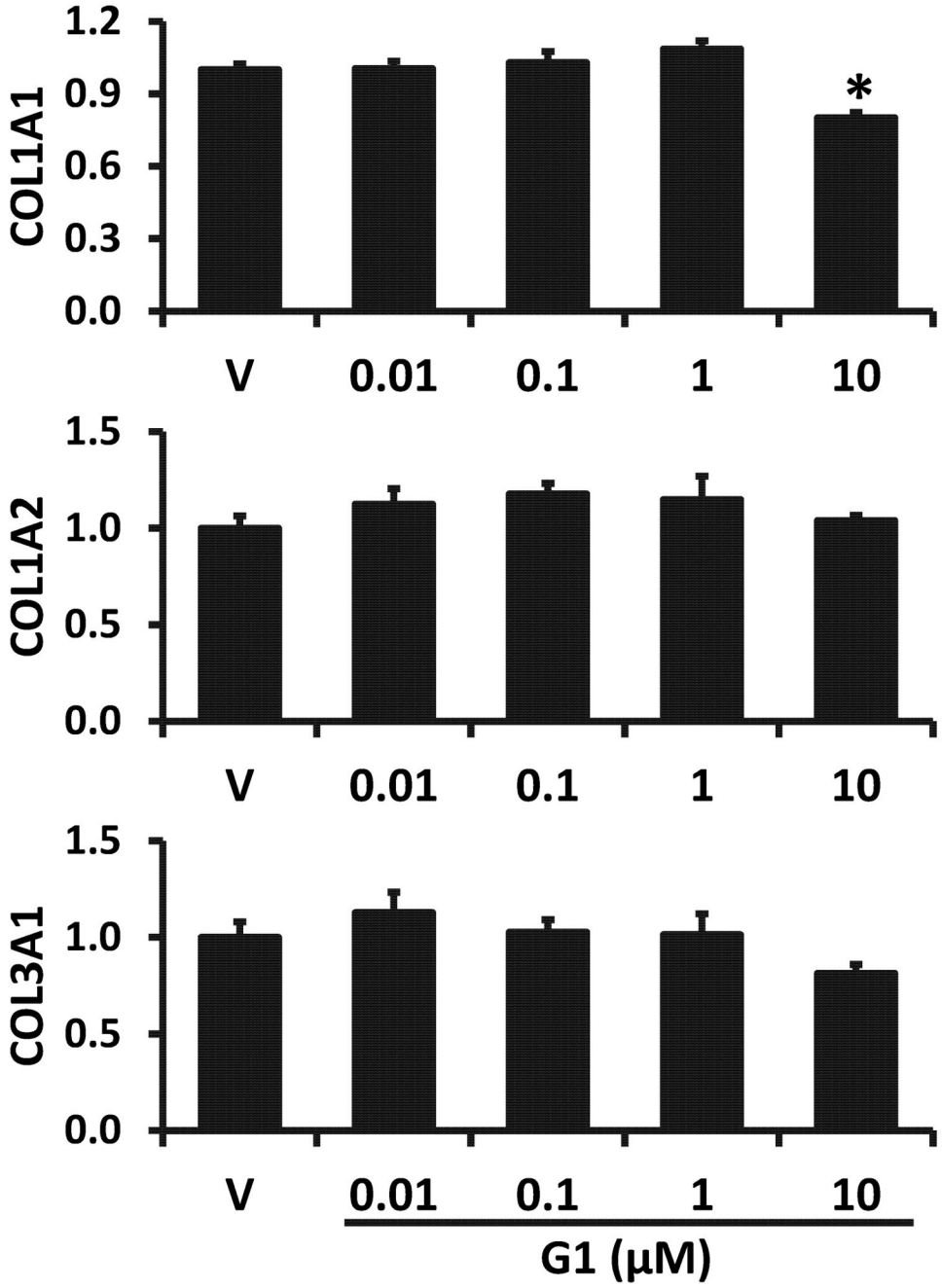


Fig. 5. GPR30 knockdown in cardiac fibroblasts

(A) GPR30 mRNA level in the GPR30 siRNA transfected cells. Values are mean ± SEM; n=4; * P<0.05 vs. GPR30-intact cells. (B) Western blot analysis showing GPR30 protein expression in GPR30 siRNA knock down vs. control siRNA cells (n=2). (C) BrdU incorporation assay in GPR30-KO versus GPR30-intact cells with or without G1 treatment. Values are mean ± SEM; n=4; * P<0.05 vs. vehicle; # P<0.05 vs. GPR30-intact cells with the same treatment. (D) CDK1 and (E) cyclin B1 mRNA levels in GPR30-KO versus GPR30-intact cells, determined by real-time PCR. Values are mean ± SEM; n=4; * P<0.05 vs. vehicle; # P<0.05 vs. GPR30-intact cells with the same treatment. (F) Western blot analysis showing CDK1 and cyclin B1 protein levels in GPR30-KO versus GPR30-intact cells treated with G1 or vehicle. Values are mean ± SEM; n=3; # P<0.05 vs. GPR30-intact cells.



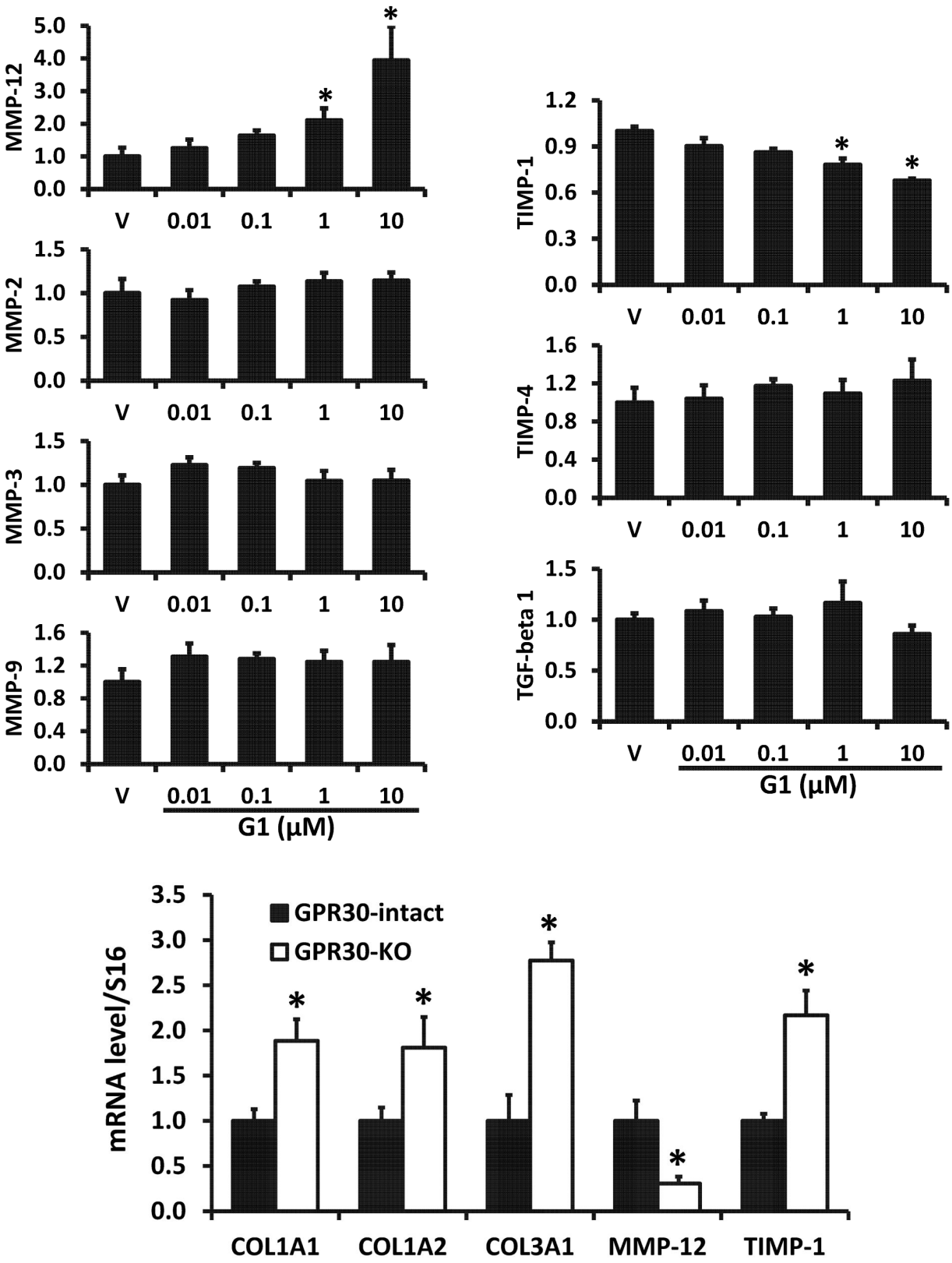


Fig. 6. Collagen related genes expression in cardiac fibroblasts

(A) COL3A1, COL1A2, and COL1A1 mRNA levels in cardiac fibroblasts treated with various doses of G1 for 24 hours. Values are mean ± SEM; n=4; * $P<0.05$ vs. vehicle. (B) MMPs, TIMPs, and TGF-β1 mRNA levels in cardiac fibroblasts treated with various doses of G1 for 24 hours. Values are mean ± SEM; n=4; * $P<0.05$ vs. vehicle. (C) COL3A1, COL1A2, COL1A1, MMP-12, and TIMP-1 mRNA levels in GPR30-KO versus GPR30-intact cells. Values are mean ± SEM; n=4; * $P<0.05$ vs. GPR30-intact cells.

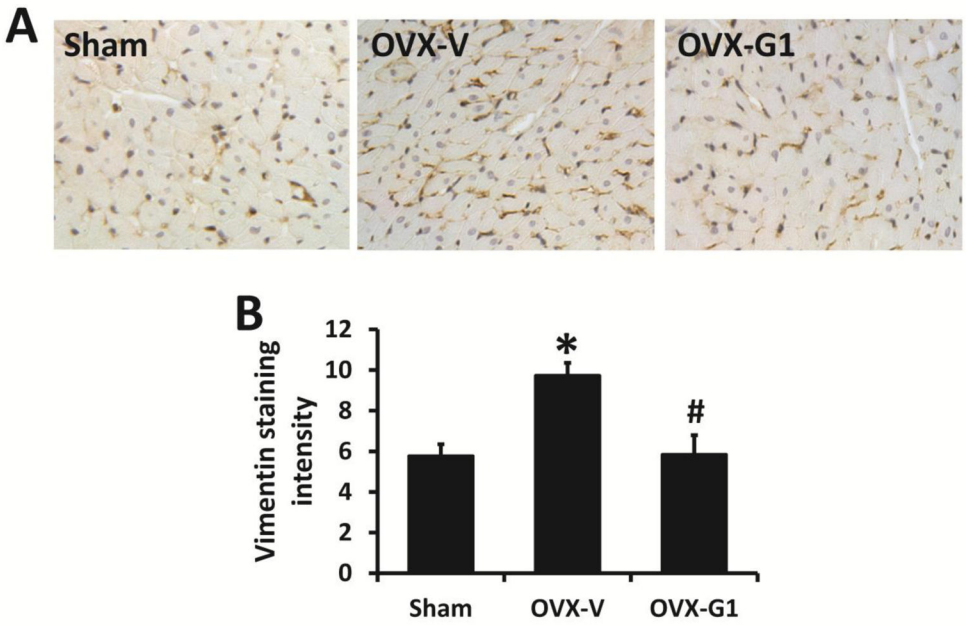


Fig. 7. Cardiac vimentin expression in sham-operated and ovariectomized female mRen2.Lewis rats treated with vehicle or G1

(A) Representative images of vimentin staining by immunohistochemistry in the left ventricles of sham and OVX-mRen2.Lewis rats. (B) The intensities of cardiac vimentin staining are quantified using NIH ImageJ software. Values are mean ± SEM; n=5; * $P<0.05$ vs. sham, # $P<0.05$ vs. OVX-V.

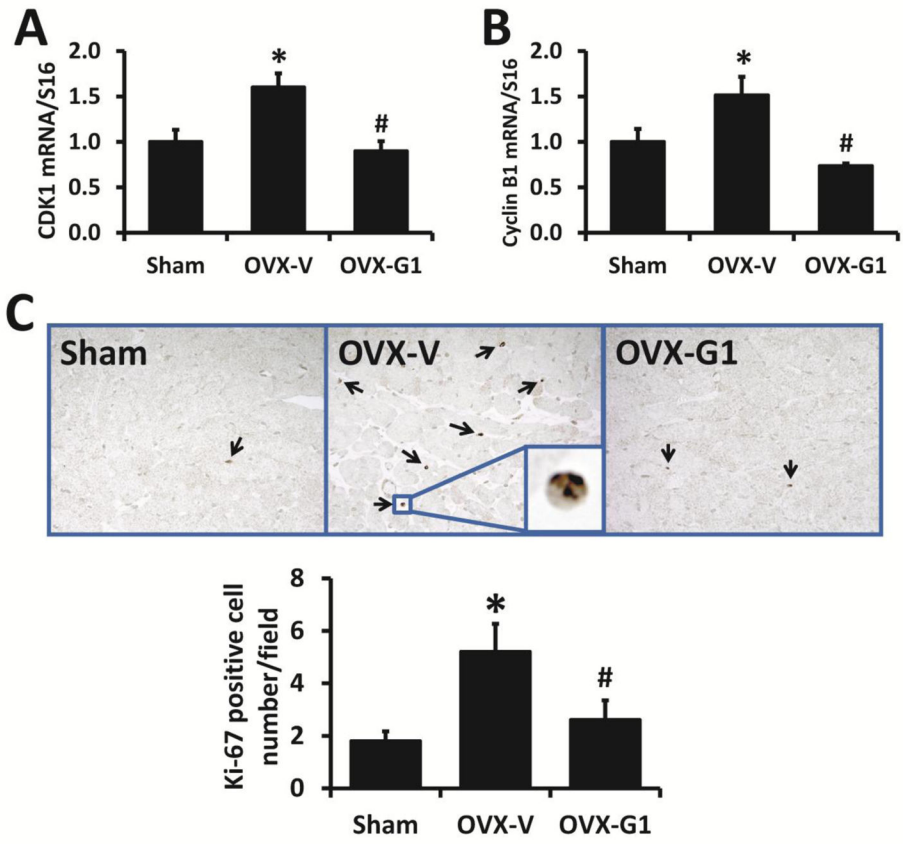


Fig. 8. Cardiac CDK1, cyclin B1, and Ki-67 expression in ovariectomized mRen2.Lewis rats

(A) Cardiac CDK1 and (B) cyclin B1 mRNA levels were determined using real-time PCR in left ventricles of sham-operated (n=10) and ovariectomized female mRen2.Lewis rats treated with vehicle (n=9) or G1 (n=7) for 2 weeks. (C) Representative images of Ki-67 staining (5 rats in each group) by immunohistochemistry in the left ventricles of mRen2.Lewis rats and the quantification showing G1 inhibited the increase of Ki-67 positive cells induced by estrogen loss. Values are mean ± SEM; * $P<0.05$ vs. sham, # $P<0.05$ vs. OVX-V.