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# Fibronectin and transforming growth factor beta contribute to erythropoietin resistance and maladaptive cardiac hypertrophy

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ABSTRACT

The use of recombinant human erythropoietin (rhEPO) to promote repair and minimize cardiac hypertrophy after myocardial infarction has had disappointing outcomes in clinical trials. We hypothesized that the beneficial non-hematopoietic effects of rhEPO against cardiac hypertrophy could be offset by the molecular changes initiated by rhEPO itself, leading to rhEPO resistance or maladaptive hypertrophy. This hypothesis was investigated using an isoproterenol-induced model of myocardial infarct and cardiac remodelling with emphasis on hypertrophy. In h9c2 cardiomyocytes, rhEPO decreased isoproterenol-induced hypertrophy, and the expression of the pro-fibrotic factors fibronectin, alpha smooth muscle actin and transforming growth factor beta-1 (TGF-β1). In contrast, by itself, rhEPO increased the expression of fibronectin and TGF-β1. Exogenous TGF-β1 induced a significant increase in hypertrophy, which was further potentiated by rhEPO. Exogenous fibronectin not only induced hypertrophy of cardiomyocytes, but also conferred resistance to rhEPO treatment. Based on these findings we propose that the outcome of rhEPO treatment for myocardial infarction is determined by the baseline concentrations of fibronectin and TGF-β1. If endogenous fibronectin or TGF-β levels are above a certain threshold, they could cause resistance to rhEPO therapy and enhancement of cardiac hypertrophy, respectively, leading to maladaptive hypertrophy.



## 1. Introduction

Chronic heart failure (CHF) is the leading cause of morbidity and mortality in the Western world [1]. CHF can be the direct result of cardiovascular disease (CVD) such as myocardial infarction, hypertension and anemia, or the indirect result of distant organ malfunction, for example, chronic kidney disease. Irrespective of the primary etiology, to compensate for the pressure and volume overload imposed by CVD, the heart undergoes adaptive remodelling and becomes hypertrophic [2]. Although the initial hypertrophy is an adaptive mechanism of the heart to compensate for the stress induced by CVD, sustained hypertrophy leads to maladaptive hypertrophy and eventual heart failure [2,3]. It is now well-established that cardiac hypertrophy is an independent risk factor for nearly all forms of heart failure and that prevention of hypertrophy is an important therapeutic target to prevent heart failure [2,3].

One common characteristic of CHF, irrespective of the aetiology, is anemia. Chronic untreated anemia is an independent risk factor for the development of cardiac hypertrophy and subsequent CHF [4]. Recombinant human erythropoietin (rhEPO) has been successfully used to treat patients with chronic anemia. The biological effects of rhEPO are mediated through interactions with its receptor, EPOR. Although the amelioration of anemia by rhEPO is largely attributed to its hematopoietic effects, for example, normalization of hemoglobin and hematocrit levels [5,6], one remarkable finding is that EPO also improves cardiac hypertrophy in anemic patients, irrespective of the primary etiology of hypertrophy. These observations suggested a non-hematopoietic role for EPO beyond the correction of anemia [6]. This notion was strengthened by the identification of EPOR in many non-hematopoietic cells including cardiomyoblasts and endothelial cells [7,8].

Consequently, the non-hematopoietic effects of EPO have been extensively investigated, and animal studies have consistently concluded that EPO offers protection against cardiac hypertrophy and myocardial infarction in various models of CVD [9–11]. However, the results of clinical trials have been equivocal. In particular, the

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phase II Reduction of Infarct Expansion and Ventricular Remodeling With Erythropoietin After Large Myocardial Infarction (REVEAL) trial did not demonstrate any reduction in infarct size [12]. Furthermore, in a subset of patients, there was an increase in infarct size and worse outcome in response to EPO [12]. These findings prompted an editorial in JAMA questioning the efficacy of EPO for myocardial infarction and cautioning further clinical trials [13].

Although many factors including study design, EPO dosage, time and duration of intervention have been identified as major factors, the possible molecular mechanisms behind discrepancies between animal studies and human clinical trials have not been fully explored. We hypothesize that the beneficial non-hematopoietic effects of rhEPO against cardiac hypertrophy could be offset by the molecular changes initiated by rhEPO itself, leading to rhEPO resistance or maladaptive hypertrophy. Identifying such potential molecular mechanisms could enable the development of better therapeutic strategies, as well as the efficient use of EPO. In this study we test this hypothesis on isoproterenol-induced cardiac remodelling with emphasis on hypertrophy.

## 2. Materials and methods

### 2.1. Culture medium and chemicals

Dulbecco's modified Eagle's medium (DMEM) containing F12 (1:1 DMEM/F12), fetal bovine serum (FBS), penicillin, streptomycin and Trypsin–EDTA were purchased from Gibco (Invitrogen, Carlsbad, CA). Isoproterenol, hematoxylin, eosin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and ponceau S solution were purchased from Sigma–Aldrich (St. Louis, MO). rhEPO (EPREX solution 10000 U/ml) was from Janssen-Cilag, Sydney, Australia.

### 2.2. Antibodies

Antibody for transforming growth factor beta (TGF-β; sc-146) was purchased from Santa Cruz Biotechnology (CA, USA). Antibody for fibronectin (A0245) was from Dako (Dako Australia, VIC, Australia) and alpha smooth muscle actin (α-SMA; A2547) was from Sigma–Aldrich (St. Louis, MO, USA). Secondary antibodies were from Molecular Probes (Life Technologies, Carlsbad, CA, USA).

### 2.3. Cell lines and culture

Rat myocardial cell line H9c2 was obtained from American Type Culture Collection (ATCC). The cells were cultured in DMEM/F12 containing 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 mg/ml) in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C.

### 2.4. Measurement of cell surface area as a marker of hypertrophy

Changes in surface area of cells, as a marker of hypertrophy, were analysed using a modification of a previous report [14]. In brief, the cells were seeded on glass cover slips in 24-well plates at a density of $4 \times 10^4$ cells/ml. 24 h later, the cells were incubated in culture medium containing 1% FBS with or without isoproterenol (ISO; 10 μM) and rhEPO (50, 100 or 200 U/ml). The compounds were prepared fresh in culture medium. 48 h after the treatment, the cells were washed in PBS and fixed for 20 min at room temperature in 4% formaldehyde. The cells were washed and permeabilized with 0.1% Triton X-100 for 5 min. After washing the cells in PBS, the cells were stained with hematoxylin and eosin (H&E), dehydrated in ethanol, cleared in xylene and mounted on glass slides with Depex mounting medium. The cells were viewed under Nikon Eclipse 50i microscope (Nikon Instruments Inc., NY, USA) at ×200 magnification, and four random fields from each slide were photographed. The surface areas of cells from each field were determined using NIS-Elements software version Br.

### 2.5. Cell viability assay

The cells ($5 \times 10^3$ cells/well/100 μl) were seeded in 96-well plates. 24 h later, the cells were incubated in culture medium containing 1% FBS with or without isoproterenol (ISO; 10 μM) and rhEPO (50 U/ml) and incubated in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. 48 h after the treatment, the culture medium was removed and 100 μl of fresh culture medium (without ISO or rhEPO) containing MTT (0.5 mg/ml) was added to each well and incubated in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C for 90 min. The medium was removed and 100 μl of dimethyl sulfoxide (DMSO) were added to each well to dissolve the purple formazan crystals. The absorbance was read at 570 nm with a background correction of 690 nm in a Multiscan Go Microplate Reader (Thermo Scientific, Waltham, MA, USA). The percentage of cell viability was calculated relative to the control wells, which were designated as 100%.

### 2.6. Western blotting

Cells were grown to approximately 80% confluence in 100 mm petri dishes and treated with ISO (10 μM), EPO (50 U/ml) or both. 48 h after treatment, the culture medium was removed and the cells were washed twice in ice-cold PBS. Whole cell lysates were prepared by lysing the cells in radio-immuno precipitation assay (RIPA) buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). The lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was collected and the protein content measured using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA). The lysates were aliquoted and stored at −80 °C until further use. The proteins (50 μg) were resolved in 10% Tris–HCl gel and electro-transferred into polyvinylidene fluoride membranes (Millipore Corporation, MA, USA). Equal loading of proteins was confirmed by staining the membrane with Ponceau S solution. Standard Western blotting procedures were followed, and the signals were detected by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). The differences in intensities of the signals were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD).

### 2.7. Data analyses

The results are expressed as mean ± standard error (SE) of mean. Comparisons between groups were performed by analysis of variance (ANOVA) with Tukey's post hoc test or Student's *t*-test, where appropriate. Analyses were performed using Graphpad Instat software (San Diego, CA, USA). $p < 0.05$ was considered significant.

## 3. Results

### 3.1. EPO reversed isoproterenol-induced hypertrophy of cardiomyocytes

Treatment of cardiomyocytes with ISO (10 μM) induced a significant increase in hypertrophy as evidenced by increases in the surface areas of the whole cells (Fig. 1 A) and the nuclei at 48 h (Fig. 1B). A representative H&E stained cells from each group is shown in Fig. 1 C. Co-treatment with EPO (50, 100 and 200 U/ml)

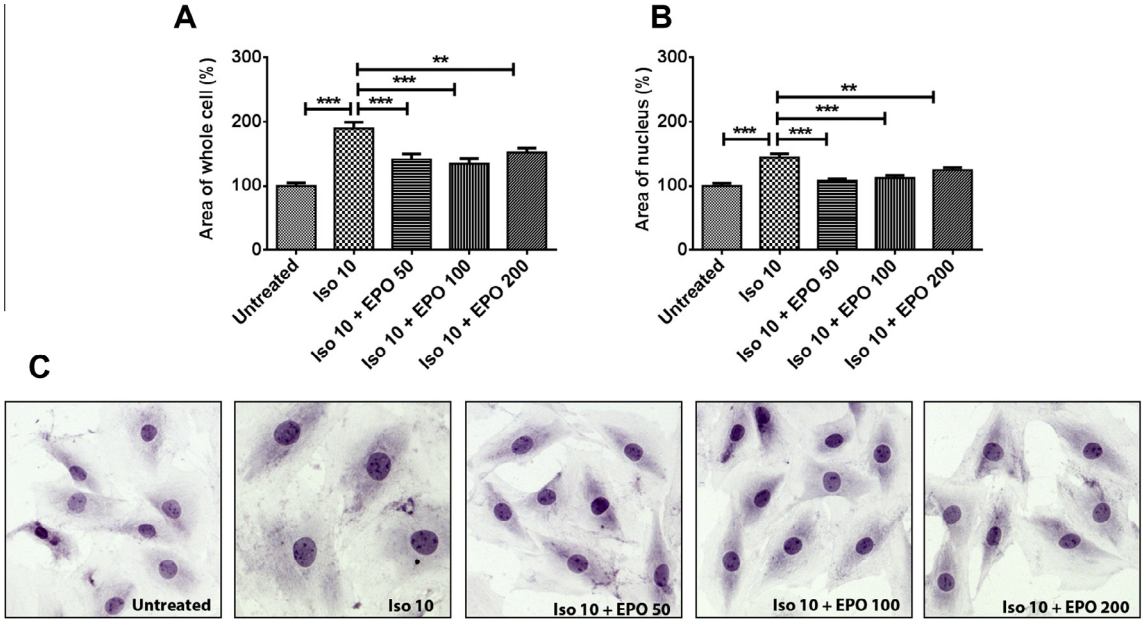


**Fig. 1.** EPO attenuated isoproterenol-induced hypertrophy of cardiomyocytes. At 48 h, EPO significantly attenuated isoproterenol-induced increases in the surface areas of whole cells (A) and the nuclei (B). A representative H&E photomicrograph is shown in C. Original magnification ×200. Iso 10 = isoproterenol 10 μM; EPO 50 = erythropoietin 50 U/ml; EPO 100 = erythropoietin 100 U/ml; EPO 200 = erythropoietin 200 U/ml. $^{**}p < 0.01$ and $^{***}p < 0.001$.

significantly decreased ISO-induced hypertrophy (Fig. 1 A–C). Treatment of cells with vehicle for EPO or EPO (50 U/ml) alone did not induce any significant changes in hypertrophy (data not shown). Furthermore no significant changes in cell viability was observed in response to vehicle for EPO or EPO (50 U/ml) or ISO treatment (data not shown). Further studies were performed with 10 μM of ISO and 50 U/ml of EPO.

### *3.2. EPO attenuated isoproterenol-induced fibronectin and TGF-β of cardiomyocytes but increased them by itself*

There was a significant increase in fibronectin (Fig. 2 A), α-SMA (Fig. 2B) and TGF-β (Fig. 2 C) expression in response to ISO treatment. Expression of all these pro-fibrotic factors was decreased significantly by co-treatment with EPO. Of note, EPO by itself sig-

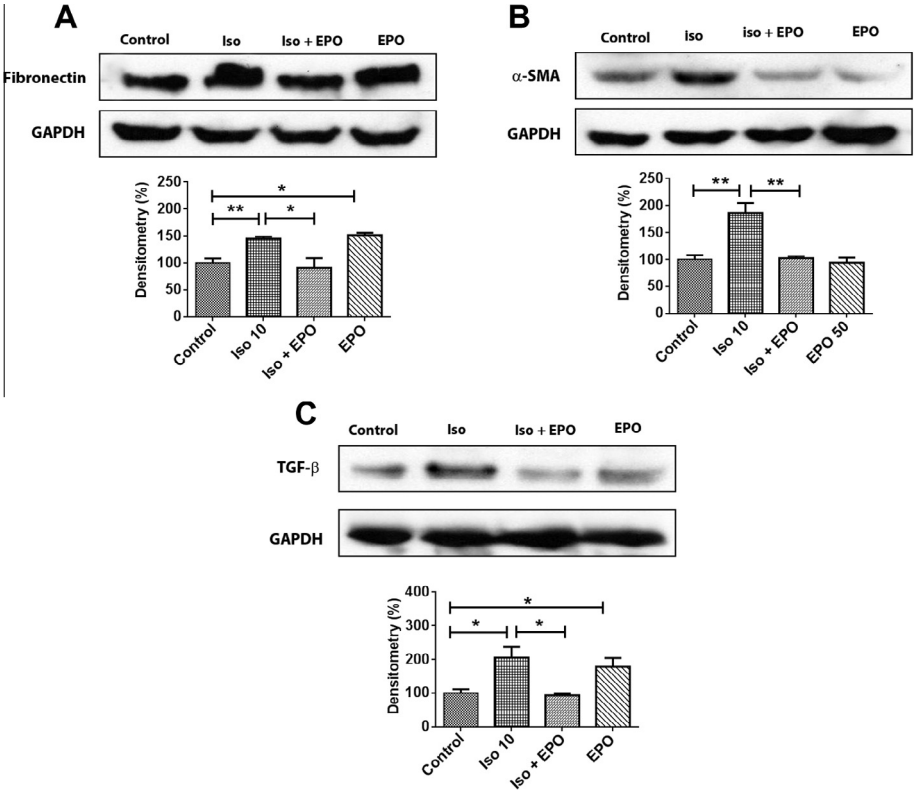


**Fig. 2.** Dual role of EPO on fibronectin and TGF-β. EPO significantly decreased isoproterenol-induced fibronectin (A), α-SMA (B) and TGF-β (C). However, EPO by itself resulted in a significant increase in fibronectin (A, last lane) and TGF-β (C, last lane) when compared with untreated controls. Iso 10 = isoproterenol 10 μM; EPO = erythropoietin 50 U/ml; $^{*}p < 0.05$; $^{**}p < 0.01$.

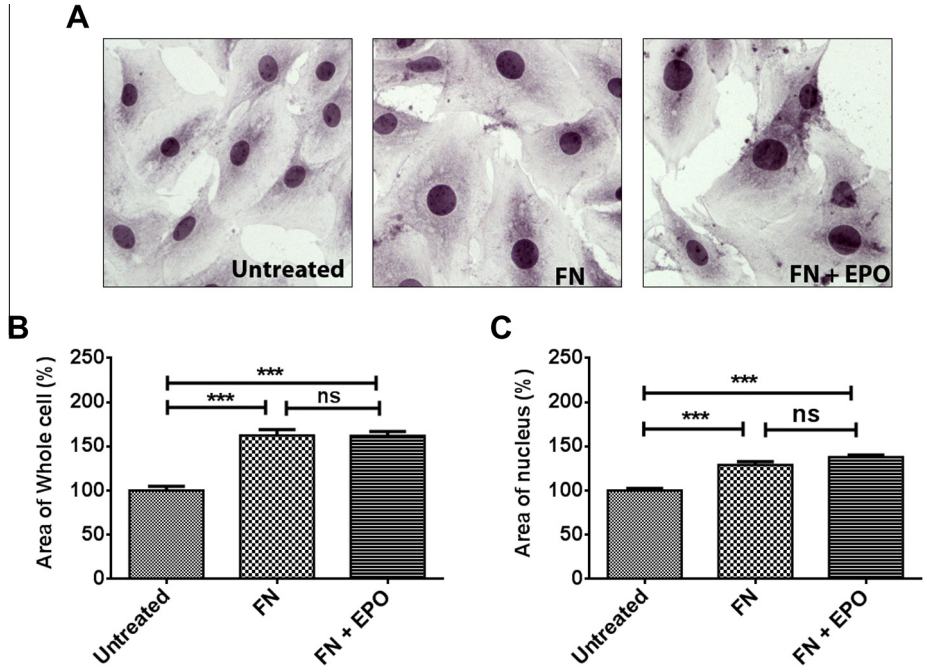


**Fig. 3.** Fibronectin-mediated resistance to EPO. Treatment of cardiomyocytes for 48 h with exogenous fibronectin (20 μg/ml) induced a significant hypertrophy (A) as evidenced by a significant increase in the surface areas of the whole cells (B) and nuclei (C). EPO failed to reverse fibronectin-induced increase in the surface areas of the whole cells (B) and nuclei (C). FN = fibronectin 20 μg/ml; EPO = erythropoietin 50 U/ml; $^{***}p < 0.001$; ns = not significant.

nificantly increased the expression of fibronectin (Fig. 2 A, last lane) and TGF-β (Fig. 2 C, last lane) when compared with untreated control.

### 3.3. Fibronectin-mediated EPO resistance

As EPO alone increased the expression of fibronectin (Fig. 2 A), we investigated the effect of exogenous fibronectin on cardiomyocytes. H9c2 cells were treated with exogenous fibronectin (20 μg/ml), and 60 min later, EPO (50 U/ml) was added. After 48 h of treatment, hypertrophy was analysed. Fibronectin induced a significant increase in hypertrophy (Fig. 3 A) as evidenced by significant increases in the surface areas of the whole cells (Fig. 3 B) and nuclei (Fig. 3 C). EPO failed to reverse these changes (Fig. 3 A–C), an indication of fibronectin-mediated resistance to EPO. Concomitant treatment of fibronectin and EPO did not reverse fibronectin-induced hypertrophy (data not shown). To test if pre-treatment with EPO would have any effects, in separate set of experiments, cardiomyocytes were pre-treated with EPO for sixty minutes before the addition of fibronectin. This also failed to reverse fibronectin-induced hypertrophy (data not shown).

### 3.4. EPO potentiated TGF-β-induced hypertrophy

As EPO alone increased the expression of TGF-β (Fig. 2 C), we tested the effect of exogenous TGF-β on cardiomyocytes. H9c2 cells were treated with TGF-β (5 ng/ml), and sixty minutes later, treated with EPO (50 U/ml). Hypertrophy was measured after 48 h of treatment. TGF-β induced a significant hypertrophy (Fig. 4 A), however, its effect was different to that of fibronectin. TGF-β significantly increased the surface areas of the whole cells (Fig. 4 B), but did not alter the surface areas of the nuclei (Fig. 4 C). EPO further potentiated TGF-β-induced hypertrophy as evidenced by a significant increase in surface areas of whole cells (Fig. 4 B). Concomitant or pre-treatment with EPO also potentiated TGF-β-induced hypertrophy (data not shown).

## 4. Discussion

Myocardial hypertrophy is often associated with an increase in plasma catecholamines [15,16]. ISO, a selective β-adrenergic receptor agonist catecholamine, is the most widely used compound experimentally to induce cardiac hypertrophy [17]. To study the non-hematopoietic effects of rhEPO on cardiac hypertrophy without the confounding effects of hematopoiesis, we used this well-established *in vitro* model. In line with previous reports [14,18,19] ISO induced a significant increase in hypertrophy, TGF-β, fibronectin and α-SMA.

TGF-β and fibronectin are essential for normal physiological processes such as embryonic development and wound healing [20–22]. However, they also contribute to pathologic ventricular hypertrophy and cardiac remodelling [21,23]. TGF-β is increased during cardiac hypertrophy and contributes to heart failure [24–26]. Fibronectin, an important component of the extracellular matrix, is increased during myocardial infarction [23,27], and induces hypertrophy of cardiomyocytes [28]. Conditional deletions of TGF-β and fibronectin prevent left ventricular hypertrophy and heart failure in various experimental models [20,21,29]. Although the role of α-SMA has not received much attention, it is increased in ISO-induced cardiac hypertrophy [19,30]. Acting in autocrine and paracrine fashions, they promote hypertrophy of cardiomyoblasts leading to fibrosis, myocardial stiffness, reduced ventricular chamber size, cell death either by apoptosis or necrosis and eventual heart failure. EPO significantly decreased ISO-induced hypertrophy, fibronectin, TGF-β and α-SMA and fibronectin, thus offering a possible explanation for its anti-hypertrophic effect. EPO has been previously shown to decrease TGF-β [31] and α-SMA [32] in various experimental models.

While these results support the non-hematopoietic effects of EPO on hypertrophy, the possible reasons for the conflicting clinical data, especially adverse clinical outcomes [12], need to be explored. Although EPO reduced the expression of ISO-induced fibronectin and TGF-β, by itself, it induced a significant increase

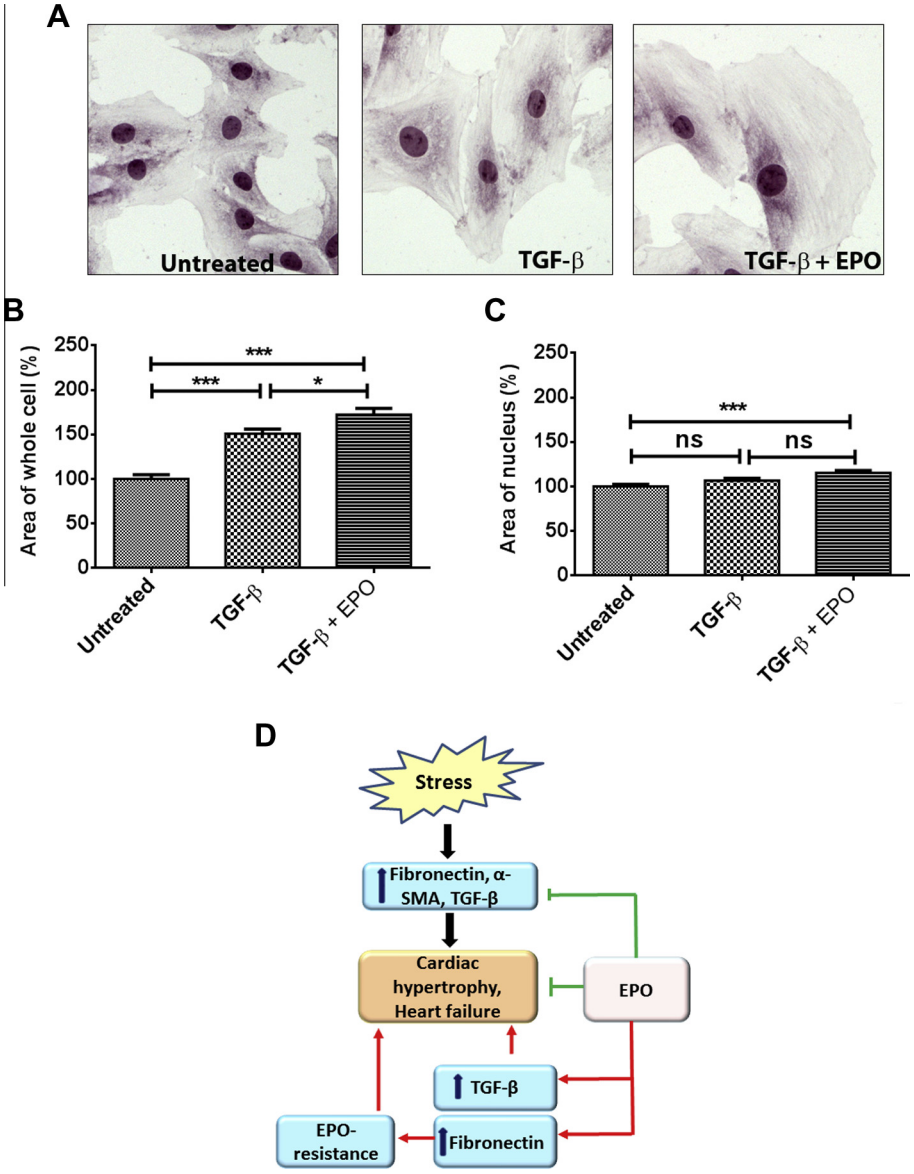


**Fig. 4.** EPO potentiated TGF-β-induced hypertrophy. Treatment of cardiomyocytes for 48 h with exogenous TGF-β (5 ng/ml) induced a significant hypertrophy (A). However, only the surface areas of the whole cells (B), not nuclei (C) were increased. Treatment with EPO further increased the surface area of the whole cells when compared with TGF-β alone (B). When compared with controls, the size of the nuclei was also significantly increased in the TGF-β plus EPO group (C). (D), Schematics of EPO action. Under stress, fibronectin, α-SMA and TGF-β are increased in cardiomyocytes. Together they lead to cardiac hypertrophy and eventual heart failure. EPO has two modes of action. It inhibits fibronectin, α-SMA and TGF-β in cardiomyocytes thus demonstrating a protective role. However, EPO itself can contribute to EPO-resistance through the up-regulation of fibronectin, or lead to maladaptive hypertrophy and heart failure through its interaction with TGF-β. $^{***}p < 0.001$; $^{*}p < 0.05$; ns = not significant.

in fibronectin and TGF-β when compared with the controls. EPO has been shown to increase fibronectin in glomerular mesangial cells and neuronal cells, [33,34] and TGF-β in skin [35]. A short-term increase in fibronectin can be beneficial to the repair process by enabling the proliferation of resident myocardial stem cells [20,21]. However, sustained fibronectin production can confer resistance to EPO as observed in this study. While EPO was ineffective against fibronectin-induced hypertrophy, it potentiated the hypertrophy induced by TGF-β. EPO has similarly been shown to enhance TGF-β in skin wounds where they exert a synergistic effect on the migration of fibroblasts, granulation tissue formation, tissue remodeling, and α-SMA expression [35]. Thus TGF-β, although needed in the first few weeks of post myocardial infarction to make sure the scar is strong, could contribute to maladaptive cardiac hypertrophy in response to EPO treatment.

Taken together, these findings apart from demonstrating the anti-hypertrophic effects of EPO, also offer a possible explanation for the inconsistent clinical trial findings. Fig. 4 D provides a summary of our hypothesis. EPO reverses hypertrophy through the inhibition of fibronectin and TGF-β. However, EPO by itself can induce these factors, offering resistance to EPO therapy through interaction with fibronectin, or potentiating the hypertrophic effects of TGF-β leading to maladaptive hypertrophy and heart failure. We propose that the outcome of EPO treatment is determined by the baseline levels of fibronectin and TGF-β. If the endogenous fibronectin level is above a certain threshold, it could offer resistance to EPO therapy, and if TGF-β is above a certain threshold, it could further enhance cardiac hypertrophy. Measuring baseline fibronectin and TGF-β in cardiac patients before the administration of EPO may lead to more effective management. Further studies on TGF-β and fibronectin levels are warranted to test this proposal. Furthermore, why EPO decreases ISO-induced fibronectin and TGF-β, but increases their levels by itself also warrants further research.

## 5. Conflict of interest

Professor David Johnson is a current recipient of a Queensland Government Health Research Fellowship. He has received consultancy fees, research funds, speaking honoraria and travel sponsorships from Jannsen-Cilag, Amgen, Pfizer and Roche. All other authors verify that they have nothing to disclose.

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