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Transforming growth factor-β2 enhances differentiation of cardiac myocytes from embryonic stem cells

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Abstract

Stem cell therapy holds great promise for the treatment of injured myocardium, but is challenged by a limited supply of appropriate cells. Three different isoforms of transforming growth factor-β (TGF-β) -β1, -β2, and -β3 exhibit distinct regulatory effects on cell growth, differentiation, and migration during embryonic development. We compared the effects of these three different isoforms on cardiomyocyte differentiation from embryonic stem (ES) cells. In contrast to TGF-β1, or -β3, treatment of mouse ES cells with TGF-β2 isoform significantly increased embryoid body (EB) proliferation as well as the extent of the EB outgrowth that beat rhythmically. At 17 days, 49% of the EBs treated with TGF-β2 exhibited spontaneous beating compared with 15% in controls. Cardiac myocyte specific protein markers sarcomeric myosin and α-actin were demonstrated in beating EBs and cells isolated from EBs. In conclusion, TGF-β2 but not TGF-β1, or -β3 promotes cardiac myocyte differentiation from ES cells.

Keywords: Stem cells; TGF-β2; Embryoid body; Cardiac myocytes; Heart

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the preimplantation embryo and represent pluripotent-undifferentiated cells. ES cells are capable of self-renewal as well as differentiation into all three embryonic germ layers: ectoderm, endoderm, and mesoderm [1,2]. In vitro differentiation of ES cells closely recapitulates the early embryonic development process, and this system has been used to differentiate pluripotent ES cells into a variety of specialized cells, including cardiomyocytes under appropriate conditions [1,3]. However, ES cells transplanted into the infarcted heart can differentiate into cardiomyocytes and contribute to the heart regeneration and improve heart function [4]. Such a capability of generation of functional cardiomyocytes from the human ES cells offers a great potential for cell therapy for heart failure patients [5]. However, factors that can induce selective and guided differentiation of ES cells to increase the number of functional cardiomyocytes must be elucidated.

Transforming growth factor-β (TGF-β) has been shown to induce cardiac differentiation from ES cells as well as regulate cell growth, differentiation, and migration during embryonic development [6–10]. Three different isoforms of TGF-β; β1, β2, and β3, have been identified in mammals [11]. The TGF-β isoforms of knockout mice have been shown to be phenotypically and functionally distinct for heart development. For example: TGF-β1 or TGF-β3 knockout mice have no major congenital heart defects; TGF-β2-knockout mice suffer from cardiovascular anomalies such as failure of normal completion of looping, septation of the outflow tract, and ventricular remodeling [12–16]. Moreover, these TGF-β isoforms have also been shown to generate distinguishing effects in vitro as well as ex vivo. For example: TGF-β1, but not TGF-β2, inhibits proliferation of hematopoietic progenitor cells [17]; bovine aortic

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endothelial cells (BAECs) [18]; TGF-β1, but not TGF-β2 also significantly inhibits cell migration of BAECs [18]; TGF-β2 but not TGF-β1, plays a role in mesoderm induction studied in amphibian explants [19]; TGF-β2 and not TGF-β3 has been shown to induce mouse epithelial–mesenchymal transformations in collagen gel explant cultures [20].

The objective of this study was to compare the effects of TGF-βs; β1, β2, and β3 isoforms on the induction of ES cell differentiation to form beating cardiac myocytes. In the present study, we demonstrate that treatment of mouse ES cells with TGF-β2 but not TGF-β1 or TGF-β3 induces significantly increased embryoid body (EB) proliferation as well as increases in the number of beating cardiac myocytes. The beating EBs exhibited cardiac myocyte specific proteins by immunostaining and Western blot analysis.

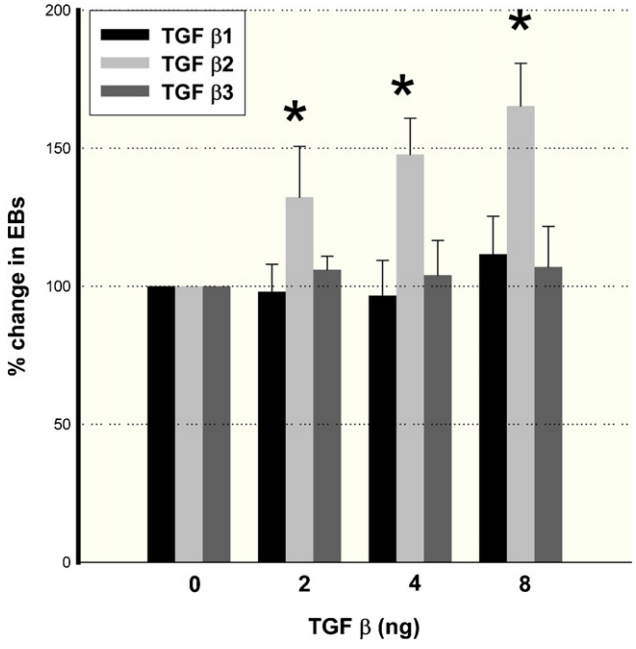


Fig. 1. Effect of TGF-β1, -β2, or -β3 on embryoid body (EB) proliferation derived from mouse embryonic stem (ES) cells. The results are plotted as a percentage increase in EBs (*$p < 0.05$ vs control) on D5. The data are from the sets of three-five independent experiments.

Materials and methods

ES cell culture and embryoid body formation. Mouse ES cell line CGR8 stably transfected with cardiac specific α-actin promoter expressing yellow fluorescence protein (YFP) was kindly provided by Michel Puceat (Centre de Researches, INSERM, U390 France). Cells were grown on a gelatin-coated (0.1% gelatin solution) tissue culture plate and were maintained without feeder cells in Dulbecco's minimum essential medium (Gibco) supplemented with sodium pyruvate, glutamine, penicillin/streptomycin, non-essential amino acids, β-mercaptoethanol, and 15% ES cell qualified fetal bovine serum. Leukemia inhibitory factor (LIF, 1000 U/ml, Chemicon) was added to the medium to prevent ES cell differentiation. To induce differentiation, ES cells were cultured without LIF in hanging drops (~500 cells per 20 μl drop) to form embryoid bodies (EBs) for 2 days (D2), and then kept in suspension for 3 days in differentiating medium treated with 0, 2, 4, and 8 ng/ml of the bio-active forms of either recombinant human TGF-β1, TGF-β2 (Chemicon) or TGF-β3 (R&D, Minneapolis, USA). The TGF-β concentrations (2–8 ng/ml) used in the present study were well within the range of TGF-βs isoform concentrations (3–50 ng/ml) used by other investigators in their cell culture studies [19,21–25]. Finally, plating was done on gelatin-coated 100 mm tissue culture dishes up to 17 days with or without TGF-β1, -β2, and -β3 treatment. The number of EB formation, proliferation, and beating EBs were counted at D3–D17. Percentage of beating area was calculated by measuring beating area divided by total EB area and multiplied with 100. Number of beats per minute was calculated from 14 to 16 EBs from each group.

Immunohistochemical staining. EBs or isolated cardiac myocytes from EBs were rinsed twice with phosphate-buffered saline (PBS) for 5 min twice. Cells were fixed and permeabilized with methanol:acetone (2:10) for 20 min at −20 °C. Cells were incubated with either primary antibody sarcomeric myosin, MF-20 (1:20) (Developmental studies Hybridoma bank), or sarcomeric α-actin (1:200) (Sigma). After washing, cells were incubated with secondary antibody Alexa Fluor 568-conjugated with IgM or IgG antibody (Molecular Probe). Nuclear staining was performed with Hoechst 33258. Negative controls were performed either by incubating with primary or secondary antibody alone. Cells were mounted with antifade medium (Vector Laboratories) and visualized using fluorescence Zeiss microscope.

Western blot analysis. Cells were washed with PBS, enzymatically dissociated with the use of trypsin/EDTA (Gibco), and finally collected in modified RIPA buffer [50 mM Tris–HCl, pH 7.4, NP-40 (1%), so-

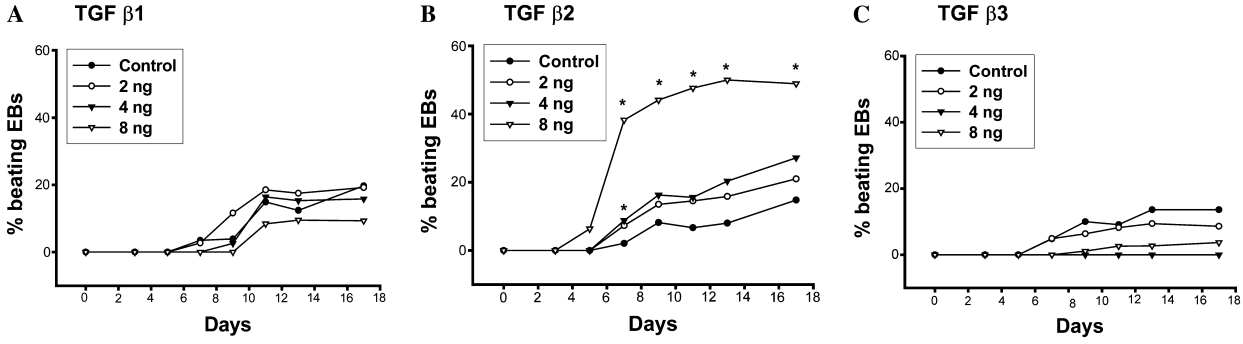


Fig. 2. Effect of TGF-β1, -β2, or -β3 on beating cardiac myocytes derived from ES–EB cell system. The results are plotted: as a percentage increase in beating EBs during D0–D17 (*$p < 0.05$ vs control). The data are from the sets of three-five independent experiments.

dium deoxycholate 0.25%, 150 mM NaCl/1 mM EDTA/2 mM sodium orthovanadate/5 mM sodium fluoride/1 mM PMSF, and mammalian protease inhibitor cocktail (Sigma)] and allowed to lyse for 30 min on ice. The cell lysates were centrifuged for 15 min at 14,000*g* at 4 °C. Protein concentration was measured in the supernatant using a Bio-Rad protein assay. Samples containing equal amounts of proteins were subjected to 10% SDS–polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (Bio-Rad). The blots were incubated with the primary antibody against sarcomeric α-actin and then with secondary antibody horseradish peroxidase anti-mouse IgG followed by detection with the chemiluminescence system, Visualizer (a gift from Upstate Cell Signaling Solutions).

Results

To compare the effect of TGF-β1, -β2, and -β3 on the induction of cardiac differentiation, CGR8 mouse ES cells that express yellow fluorescence protein under the control of a cardiac specific α-actin promoter were treated with each isoform. Cardiac α-actin is a highly specific cardiac gene expressed in the developing heart as well as cardiac myocytes derived from the ES cells [9,10]. In the present study, the EBs derived from the ES cells were treated with 0, 2, 4, and 8 ng/ml of the bio-active form of recombinant human TGF-β1, -β2 or -β3 These EBs were examined by microscopy on Day 0 (D0), D3, D5, D7, D9, D11, D13, and D17 after plating.

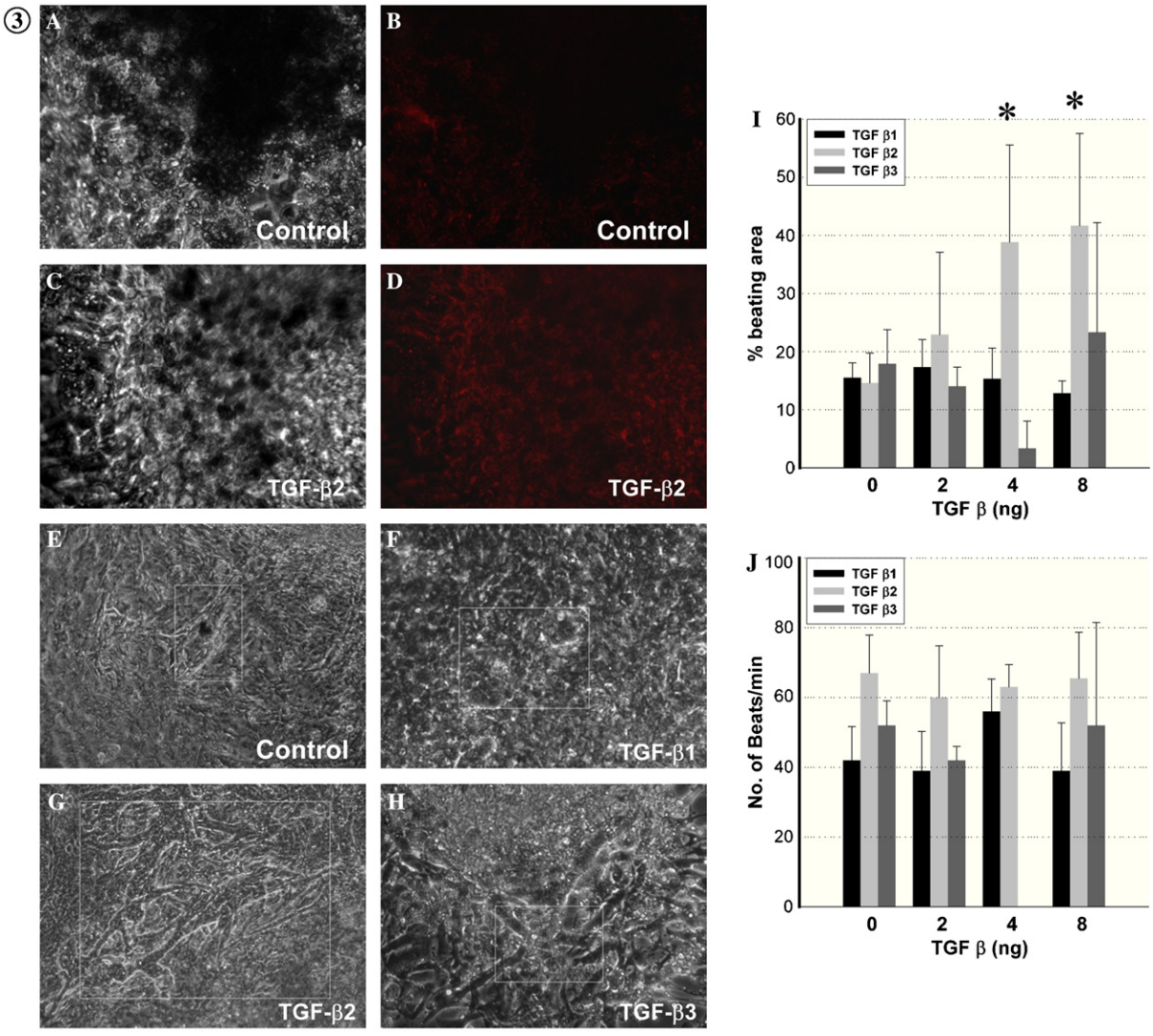
Our data demonstrate that TGF-β2 significantly increased proliferation of EB by 30–65% on D5 compared with control (Fig. 1, $p < 0.05$). Spontaneously beating cardiac myocytes were observed in rhythmically beating EBs derived from ES cells as early as D5 when treated with TGF-β2 (8 ng) (Fig. 2B, Table 1) compared with controls that did not exhibit EB beating until D7 (Fig. 2B, Table 1). The percentage of EBs that were beating after treatment with TGF-β2 (8 ng) was significantly greater (38–50%, $p < 0.05$) compared with control that ranged from 2% to 15% on D7–D17 after plating (Fig. 2B and Table 1). In contrast, treatment with TGF-β1 or TGF-β3 did not increase EB proliferation at D5 compared with control (Fig. 1). Rhythmically and spontaneously beating EBs (Fig. 2A and Table 1) and beating area (Figs. 3 E, F, H, and I) observed after treatment with TGF-β1 or TGF-β3 were to a similar extent compared with control. Although TGF-β2 increased the number of beating EBs (Fig. 2B), and beating area ($p < 0.05$ Figs. 3 E, G, and I) compared to control.

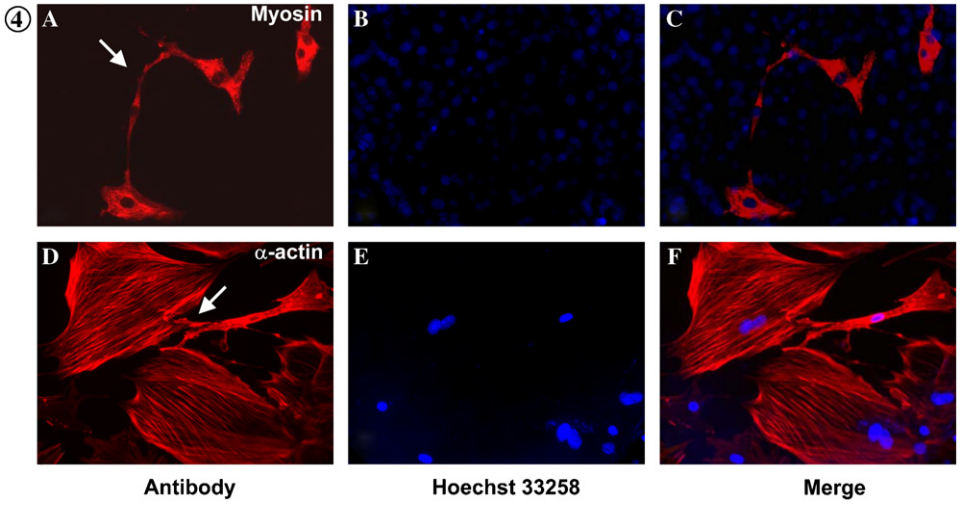
The expression of yellow fluorescence protein (YFP) that was driven by a cardiac specific α-actin promoter was increased in EBs treated with TGF-β2 compared with controls (Figs. 3A–D) but not with TGF-β1 or TGF-β3 (data not shown). The expressed YFP area indicates that there is an increased beating cardiac myocyte area. Cardiomyocytes that were treated with TGF-β2 were elongated and large in size (Fig. 3G) compared

Table 1
Percent beating cardiac myocytes after treating embryoid bodies with different TGF-β isoforms

No. of days	TGF-β1 (ng)				TGF-β2 (ng)				TGF-β3 (ng)			
	0	2	4	8	0	2	4	8	0	2	4	8
5	0	0	0	0	0	0	0	6.3 ± 4.0*	0	0	0	0
7	3.5 ± 0.36	2.7 ± 1.9	0	0	2.1 ± 1.9	7.3 ± 4.2	8.8 ± 2.8*	38.3 ± 12.3*	4.8 ± 4.6	4.9 ± 6.9	0	0
9	3.9 ± 10.7	11.6 ± 13.5	2.6 ± 3.31	0	8.2 ± 6.7	13.5 ± 5.5	16.2 ± 9.7	44.1 ± 12.7*	10.0 ± 9.4	6.3 ± 5.9	0	1.1 ± 1.6
11	14.9 ± 6.2	18.5 ± 12.1	16.4 ± 8.8	8.4 ± 9.3	6.7 ± 5.7	14.5 ± 5.0	15.6 ± 8.1	47.6 ± 13.9*	9.1 ± 8.0	8.2 ± 5.5	0	2.6 ± 1.9
13	12.4 ± 4.2	17.5 ± 11.2	15.3 ± 7.8	9.5 ± 7.4	8.0 ± 5.8	15.8 ± 5.6	20.3 ± 11.9	50.0 ± 11.9*	13.6 ± 4.9	9.4 ± 7.2	0	2.7 ± 2.0
17	19.7 ± 9.6	19.2 ± 16.9	15.8 ± 7.9	9.3 ± 7.1	14.8 ± 11.1	21.5 ± 13.3	27.2 ± 10.8	48.9 ± 11.8*	13.6 ± 4.6	8.6 ± 5.9	0	3.7 ± 2.7

Values are expressed as means ± SE.
* $p < 0.02$ vs 0 ng.


③
A
Control
B
Control
C
TGF-β2
D
TGF-β2
E
Control
F
TGF-β1
G
TGF-β2
H
TGF-β3
I
TGF β1
TGF β2
TGF β3
% beating area
TGF β (ng)
J
No. of Beats/min
TGF β (ng)


④
A
Myosin
B
C
D
α-actin
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Hoechst 33258
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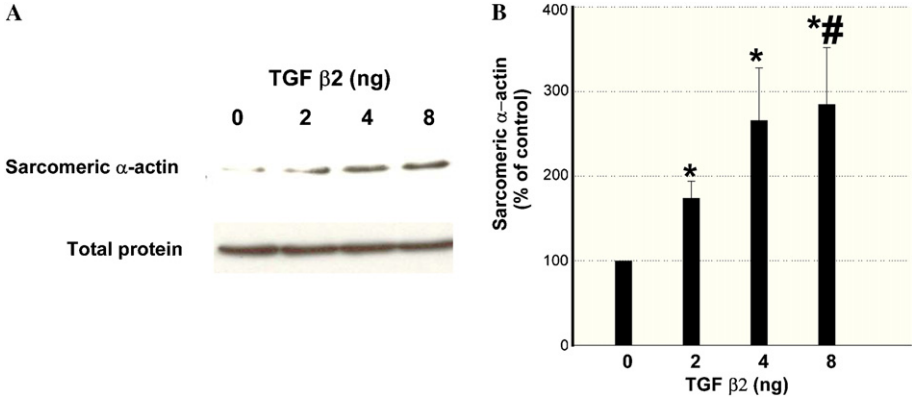

Fig. 5. Western blot analysis of TGF-β2-treated EBs shows increased expression of cardiac specific sarcomeric α-actin, and the same stripped membrane shows total loading protein (A). (B) Densitometry analysis of sarcomeric α-actin protein ($^{*}p < 0.05$ vs control (0 ng), $^{\#}p < 0.05$ vs 2 ng). Data is analyzed by setting the densitometer of the control sample as 100%. EBs treated with indicated concentrations of TGF-β2 for 17 days and Western blot was performed as described in the Materials and methods. The data are from the sets of three-four independent experiments.

with those treated with TGF-β1 or -β3 or control (Figs. 3E, F, and H). The number of beats per min was similar with either TGF-β isoforms compared to control (Fig. 3J).

Synchronously beating EBs and isolated cells exhibited characteristics of cardiac myocytes demonstrated by immunostaining with antibodies directed against sarcomeric myosin (Figs. 4A–C) and α-actin (Figs. 4D–F). Nuclei were visualized with Hoechst-33258. We quantified the effect of TGF-β2 on the protein expression of cardiac specific sarcomeric α-actin in synchronously beating EBs derived from ES cells with Western blot analysis. As shown in Fig. 5A, increased expression of cardiac protein α-actin was apparent after treatment with TGF-β2 ($p < 0.05$ by densitometry comparing 8 ng TGF-β2 with control and 2 ng) (Fig. 5).

Discussion

The generation of functional cardiomyocytes from ES cells has several potential applications including myocardial regeneration through cell transplantation [1,5]. Transplanted ES cells in the infarcted heart show limited differentiation into cardiomyocytes to regenerate the infarcted heart [4]. Therefore, the identification of the appropriate growth factor(s) to enhance cardiomyocyte formation from ES cells is needed to improve cell therapy. Growth and differentiation factors play an important role in determining the fate of the ES cell-derived cell lineages. For example: IL-3 promotes ES cell differentiation to macrophages, mast or neutrophils [26]; IL-6 to erythroid lineages [27]; TGF-β in cardiac differentiation; and retinoic acid to neurons [28].

We undertook this study to determine whether direct treatment of TGF-βs: β1, β2, or β3 isoforms would promote the differentiation of beating cardiomyocytes derived from ES-EB cell system. Our data for the first time demonstrate that treatment with TGF-β2 but not with TGF-β1 or TGF-β3 significantly enhanced the number of EB proliferation as well as spontaneous and rhythmically beating cardiac myocytes derived from the ES cells. Our observation is consistent with the previous reports indicating that TGF-β participates in cardiac differentiation and the isoforms of TGF-βs (β1, β2, and β3) generate various effects in the cell culture. For example: TGF-β1 promotes differentiation of smooth muscle cells from ES cells [29]. TGF-β2 but not TGF-β1 induces mesoderm from amphibians ex vivo [19]. TGF-β3 promotes chondrogenic differentiation of human mesenchymal stem cells [30]. Moreover, knockout of the TGF-β2 gene shows cardiovascular malformations with severe abnormalities of several cardiac segments but TGF-β1 or -β3

Fig. 3. The effects of TGF-β1, -β2, or -β3 on EB beating areas. TGF-β2-treated EBs show increased beating EB area compared with control and TGF-β1 or -β3. Embryoid bodies were formed from CGR8 ES cells driven by cardiac specific α-actin promoter expressing yellow fluorescence protein. At D9, EBs treated with TGF-β2 compared to control (A, phase; B, fluorescence) show large beating area and express large area of fluorescence (C, phase; D, fluorescence). At D17, area of beating cardiomyocytes in control (E), TGF-β1, or -β3 (F,H) (white rectangular) was much smaller than that seen in TGF-β2 treated EBs (G, white rectangular). Original magnification, 200× (A–F). The results from beating areas were also calculated and plotted as percentage of beating areas (I, $^{*}p < 0.05$ vs control) at D17. Beats per minute were also counted from these beating EBs (J). The data are from the sets of three-five independent experiments.

Fig. 4. TGF-β2-treated EBs show immunostaining for cardiac specific proteins. (A) sarcomeric MHC (red); and (D) sarcomeric α-actin (red); (B,E), show nuclei stained blue with Hoechst 33,258. Merged images of cardiac specific proteins and nuclei are shown in (C,F). Arrow indicates cell–cell attachment (A,D). Original magnification, 200× (A–C), 320× (D–F).

knockout showed no severe cardiovascular abnormalities [12–16].

Moreover, increased concentrations of TGF-β2 varing from 2 to 8 ng/ml in different experiments also increased the percentage of beating EBs (Fig. 2B, Table 1). The concentration dependent effect of TGF-β2 in the present study is in agreement with the other report which demonstrates that animal explants treated with increased TGF-β2 concentrations varied from 3 to 12 ng/ml and higher (200 ng/ml) also inducing mesoderm from amphibian explants as well as increased α-actin mRNA levels [19]. In contrast, TGF-β1 or TGF-β3 showed no concentration dependent effect for increasing beating cardiomyocytes.

These beating cardiac myocytes stained positive with two known sarcomeric myosin or α-actin cardiac specific protein markers. Moreover, Western blot analysis confirmed that TGF-β2 increases protein expression of cardiac specific α-actin. Based on these observations, we suggest that TGF-β2 derived cardiac myocytes from the ES cells are functional cardiomyocytes. TGF-β has been shown to up-regulate cardiac transcription factors and cardiomyocyte differentiation from mouse ES cells [6]. Our data demonstrate also that TGF-β2 derived cardiomyocytes were elongated and large in size. They exhibit increased beating area compared with controls (Fig. 3G). In contrast, TGF-β1 or -β3 derived cardiomyocytes were not elongated and large in size (Figs. 3F and H). These observations are also in accordance with the observations of TGF-β2 and not TGF-β1 treated animal explants which formed elongated structures [19]. Although, cell–cell junction formation, cell morphology, and sarcomeric development were not the focus of the present study. We have observed that cardiomyocytes derived from ES cells were mononucleated, were either round or elongated in shape, and there were apparent cell to cell attachment suggesting the possibility of formed cell–cell junctions (Fig. 4) and this is consistent with the previous investigations [1,31].

In conclusion, we suggest that TGF-β2 but not TGF-β1 or -3 markedly increases cardiac differentiation derived from mouse ES cells. The ES cells treated with TGF-β2 may be a useful source to produce a sufficient number of cardiac myocytes for cellular transplantation strategies and many other applications. This growth factor might have potential use for the generation of increased number of beating cardiac myocytes from human ES cells, which could have major application in the treatment of ischemic heart disease patients.

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