Effect of basic fibroblast growth factor on cardiomyocyte differentiation from mouse embryonic stem cells

Shiva Khezri, BSc, *Mojtaba R. Valojerdi*, PhD, *Houri Sepehri*, PhD, *Hossein Baharvand*, PhD.

ABSTRACT

Objective: To investigate the effect of basic fibroblast growth factor (bFGF) on the differentiation of embryonic stem cells (ESCs) into early cardiomyocytes.

Methods: Embryoid bodies (EBs) were produced from mouse ESC line (Royan B1) in hanging drops and cultured for 5 days as suspension. During the first 2 days of suspension, the EBs of the experimental group were treated with 10 ng/ml of bFGF and subsequently plated onto gelatin-coated tissue culture dishes (day 7). The differentiated cells were evaluated pharmacologically, by immunocytochemistry, and so forth. The study was carried out in the Department of Stem Cells, Royan Institute, Tehran, Iran in 2005.

Results: The beating frequency in the bFGF treated EBs was less than that in the control group. In addition, the beating in the EBs of the experimental group, treated with isoprenaline and phenylephrine, was only more than 7+3 days in comparison to the control group. The response of the EBs to carbachol was more in the bFGF group than 7+14 days. In all the stages of development, the beating cells in the EBs of both groups expressed α-actinin, myosin light chain isoform 2V, cardiac alpha-myosin heavy chain (α-MHC), and cardiac beta-myosin heavy chain (β-MHC). Nonetheless, during 7+3 days, the last 2 genes were more advanced in the bFGF group. The atrial natriuretic factor was also expressed at a late stage in both groups.

Conclusion: Basic fibroblast growth factor can only promote the early maturation of ESC-derived cardiomyocytes in terms of chronotropic characteristics and expression of cardiac α-MHC and β-MHC.

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From the Department of Stem Cells (Khezri, Baharvand) Royan Institute, Department of Biology (Khezri, Sepehri) Faculty of Science, Tehran University, Department of Anatomy (Valojerdi), School of Medical Sciences, Tarbiat Modarres University and the Department of Embryology (Valojerdi), Royan Institute, Tehran, Iran.

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Address correspondence and reprint request to: Dr. Mojtaba R. Valojerdi, Department of Embryology, Royan Institute, PO Box 19395-4644, Tehran, Iran. Tel. +98 (21) 22413790. Fax. +98 (21) 22414532. E-mail: mr_valojerdi@yahoo.com

Embryonic stem cells (ESCs), the undifferentiated cells of early embryos are extracted as permanent lines and are characterized by their self-renewal capacity and pluripotency.[1,2] These cells are able to differentiate into different cells including cardiomyocytes. The in vitro differentiation of ESCs into cardiomyocytes is very important in studying the development of the heart as it can give researchers a better understanding of the role of growth factors, transcription factors, and cardiogenesis signaling pathways in cardiomyocyte development. In vivo investigations indicate that the development of uncommitted mesoderm pre-cardiac cells into early cardiac cells is regulated by growth factors.[3] These growth factors, formed of bone morphogenetic protein-2 (BMP-2),[4] transforming growth factor-β (TGF-β), erythropoietin (EPO)[5,6] and fibroblast growth factor (FGF),[7,8] are secreted either from the anterior primitive endoderm[9] or anterior lateral regions of the embryo.[10] The positive effect of precardiac endoderm/mesoderm was shown on cardiac myocyte differentiation in murine ESCs.[11] In vitro investigations also point to the possible role of growth factors in the development of cardiomyocytes. Treatment of mouse ESCs with BMP-2 and TGF- β, revealed an increase in cardiac differentiation with a significant increase in beating areas and myofibrillogenesis.[12] Schuldiner et al[13] studied the effect of 8 growth factors including the basic FGF on the differentiation of human ESCs at the early stages of cardiogenesis. Expressed early in cardiac precursor cells, this growth factor has a direct effect on the development of cardiomyocytes.[7] Mima et al[14] suggested that FGF and its receptor in cardiac precursor cells regulated cardiomyocyte growth during the tubular stages of cardiogenesis in the chick embryo. In birds and mammals, however, cardiomyocytes terminate mitotic activity in the neonatal period, and the regeneration of cardiac muscle does not occur after myocardial injuries in adult hearts.[14] Therefore, the

identification of soluble growth factors, transcription factors, and signaling cascades capable of primary selective differentiation of ESCs into cardiac cells is a crucial issue for our understanding of cardiogenesis as well as for the development of stem-cell based therapy of cardiovascular diseases.[15]

The present study investigated the possible biological role of basic fibroblast growth factor (bFGF) as a member of the FGF family in the differentiation of mouse ESCs into cardiomyocytes. The chronotropic characteristics and expression of several cardiac- specific genes in different developmental stages were also investigated.

Methods. The study was carried out in the Department of Stem Cells, Royan Institute, Tehran, Iran in 2005. The ESC line Royan B1[16] derived from C57BL/6 mouse strain was used throughout the present study. Embryonic stem cells were kept in an undifferentiated, pluripotent state using a mitomycin C (Sigma, M0503)-inactivated feeder layer of primary cultures of mouse embryonic fibroblasts. They were cultivated on gelatin (0.1%, Sigma, G2500) coated plastic flasks (Falcon) in ESC medium containing Dulbecco's modified Eagle's medium (DMEM, Gibco, 10829-018) supplemented with 15% fetal calf serum (Gibco, 16141-079), 0.1 mM beta-mercaptoethanol (Sigma, M7522), 2 mM glutamine (Gibco, 15039-027), 0.1 mM non-essential amino acids (Sigma, M7145) and 1000 iu/ml leukemia inhibitory factor (LIF, Chemicon, ESGRO, ESG1107). The ESCs were spontaneously differentiated into beating cardiomyocytes as previously described.[17] Briefly, the main steps of differentiation included cultivation of a definite number of cells (800) in 20 µl "hanging drops" to produce embryoid bodies (EBs) for 2 days followed by cultivation, as a suspension in bacterial dishes (Griner, Germany; 628-102) for 5 days. The EBs in the experimental group were treated with bFGF (10 ng/ml, 13256029, Invitrogen, UK) for the first 2 days. The EBs were plated on 0.1% gelatin coated 24-well plates (TPP, Switzerland) for 19 days. In EB outgrowths, cardiomyocytes appeared in the form of spontaneously contracting cell clusters. To evaluate the pharmacological response of ESC-derived cardiomyocytes, the EBs with more than 10% of the outgrowths of spontaneous beating cardiomyocytes in both control and bFGF groups were treated with a 10^{-5} M concentration of isoprenaline, β_1-adrenergic receptors agonist (I-5879, Sigma, Germany), phenylephrine, α_1-adrenergic receptors agonist (P-6126, Sigma, Germany), carbachol and muscarinic cholinoceptor agonist (G-4382, Sigma, Germany) at 3 distinct developmental stages: early differentiation phase, which was shortly after the initiation of contractions (7+3 days); intermediate phase (7+7 days); and terminal differentiation phase (7+14 days) as previously described by Maltsev et al[17] The change in the number of beats per minute was calculated by counting the pulsation rates of the ESC–derived cardiomyocytes before and 3 minutes after the addition of drugs. To assess the cardiac-specific gene expression, total ribonucleic acid (RNA) from the ESC-derived cardiomyocytes on days 7+3 and 7+14 in both control and bFGF groups was extracted by means of the RNX™ (plus) kit (Cinagen, Iran) in accordance with the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was synthesized from 5µl of RNA using Revert Acid™ H Minus first strand cDNA synthesis (K- 1632, Fermentas, Germany). The cDNA samples were subjected to polymerase chain reaction (PCR) amplification with a selection of primers for mouse cardiac-related genes, including primer sets used in such amplification reactions as atrial natriuretic factor (ANF) 5'-TGATAGATGAAGGCAGGAAGCCGC-3' (forward) and 5'-GGATTGGAGCCCAGAGTGGACTAGG-3' (reverse); cardiac alpha-myosin heavy chain (α-MHC) 5'-CTGCTGGAGAGGTTATTCCTCG-3'. (forward) and 5'GGAAGAGTGAGCGGCGCATCAAGG-3' (reverse); cardiac beta-myosin heavy chain (β-MHC) 5'-TGCAAAGGCTCCAGGTCTGAGGGC-3'.(forward) and 5'-GCCAACACCAACCTGTCCAAGTTC-3' (reverse); myosin light chain isoform 2V (MLC-2V) 5'-TGTGGGTCACCTGAGGCTGTGGTTCAG-3'_ (forward) and 5'-GAAGGCTGACTATGTGTCCG-GGAGATGC-3' (reverse); and Oct-4 (marker of ESCs) 5'-GGCGTTCTCTTTGGAAAGGTGTTC-3' _(forward) and 5'-CTCGAACCACATCCTTCTCT-3' _(reverse). β-Tubulin 5'-GGAACATAGCCGTAAA-CTGC-3' (forward) and 5'-TCACTGTGCCTGAACT-TACC-3'. (reverse) were used as internal standards.[18] For each reaction, the number of the cycles was optimized so as to ensure that the signal accumulated within the linear range of the amplification. Amplification was achieved by (1) primary denaturation 93°C, 5 min; (2) denaturation 93°C, 45 sec; (3) annealing (α-MHC: 64°C, β-MHC: 64°C, MLC-2V: 60°C, ANF: 64°C, Oct 4: 70°C and β–tubulin: 60°C), 45 sec; (4) extension 72°C, 45 sec, 35 cycles of 1 to 4; and (5) final extension 72°C, 10 min. The PCR products, having been size-fractionated by 1.5% agarose gel electrophoresis, were stained with ethidium bromide before being visualized and photographed on a ultraviolet transluminator (Uvidoc, UK).

To observe the immunocytochemistry, the beating areas of 5 EBs in both control and bFGF groups, mechanically isolated with a mouth-controlled pipette at 2 developmental stages of early differentiation phase (7+3 days) and terminal differentiation phase (7+14

days), were washed in Ca^{2+} and Mg^{2+}-free phosphate buffer solution (PBS) prior to incubation with a trypsin/EDTA solution (Gibco, 15305-014) for 3 minutes. The isolated cells were resuspended in a cultivation medium and were plated on a gelatin-covered 4-well tissue culture plate at a low density for 48 hours. The contracting cells were then rinsed 2X with PBS and were fixed with methanol/acetone (3:1) at –20^{O}C. After fixation, single-cell preparations were washed several times in 0.1 M PBS and were further treated with 0.1% Trition X-100. After they were subsequently treated with 10% goat serum (16210-064, Gibco, UK) in PBS for one hour, the cells were incubated with a primary antibody, monoclonal anti α-actinin (A7811, Sigma, Germany), at a dilution of 1:800 at 37^{O}C in a humid chamber for 45 minutes. At the end of the incubation time, the cells were rinsed 3× with PBS and were incubated with fluorescence isothiocyanate (FITC)- conjugated goat anti-mouse immunoglobulin G (Sigma, F 9006, Germany) as a secondary antibody at a dilution of 1:100 in PBS at 37^{O}C for one hour. After rinsing 3× with PBS, the cells were analyzed under a fluorescent microscope (Nikon, Japan).

For statistical analysis, all the experiments were performed at least 5 times. The data of beating frequency/min and chronotropic responses were expressed as mean ± standard error of mean in both control and bFGF groups and were analyzed by Student's t test or Mann Whitney. The Statistical Package for Social Sciences version 10 was used for statistical analysis. Where p value <0.05 was considered statistically significant.

Results. The timing of the onset of spontaneous beating in the EBs of both groups was similar; it appeared early on the first day of plating. The mean of beating frequency in each EB of both groups was also similar on days 1 and 3 of plating. From day 5 onward, however, it began to reduce noticeably in the experimental group (p<0.01) (**Figure 1a**). Furthermore, the mean beating frequency in each EB of both groups changed at the 3 stages of cardiomyocyte development with the administration of cardioactive drugs such as isoprenaline (**Figure 1b**), phenylephrine (**Figure 1c**), and carbachol (**Figure 1d**).

The changes in beating frequency recovered several hours after the removal of the drugs, showing the

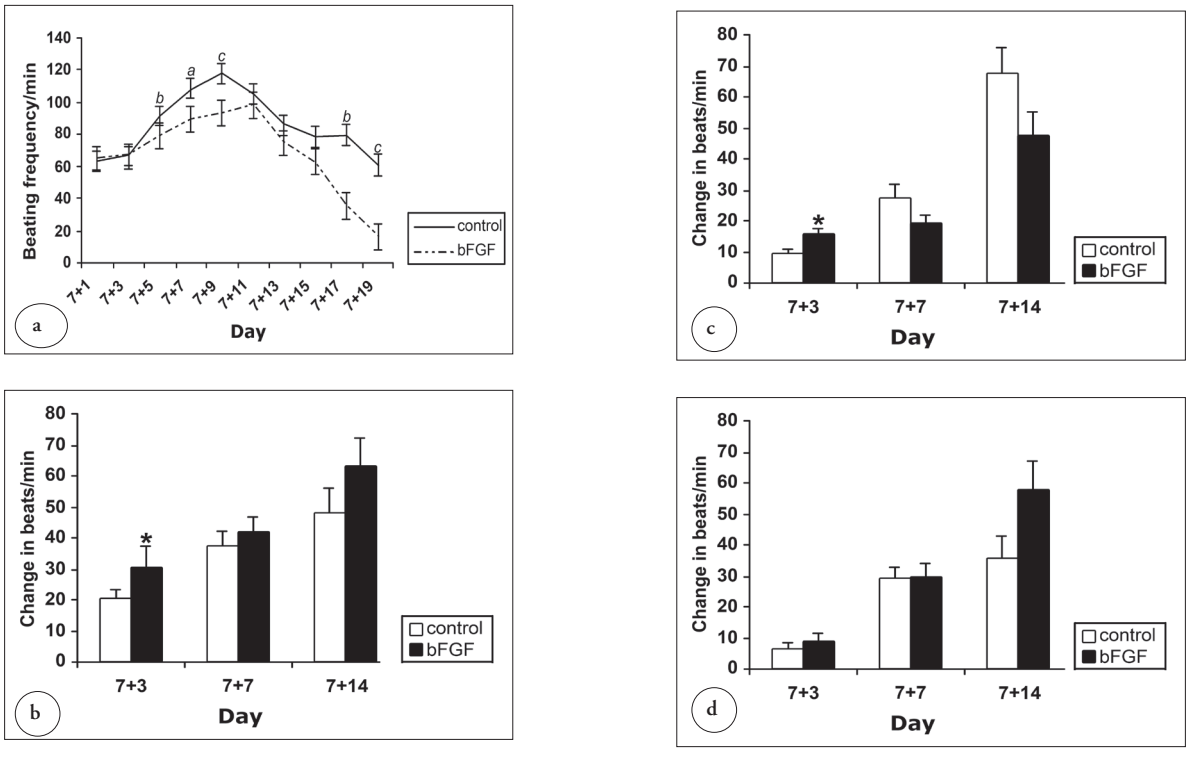

Figure 1 - Beating frequency and pharmacological responses of embryonic stem cells-derived cardiomyocytes. (**a**) Beating frequency during differentiation, a: p<0.05, b: p<0.01, c: p<0.001. Chronotropic effects of isoprenaline (**b**), phenylephrine (**c**), and carbachol (**d**) at early (7+3), intermediate (7+7), and late (7+14) stages in both control and basic fibroblast growth factor groups, * p<0.05

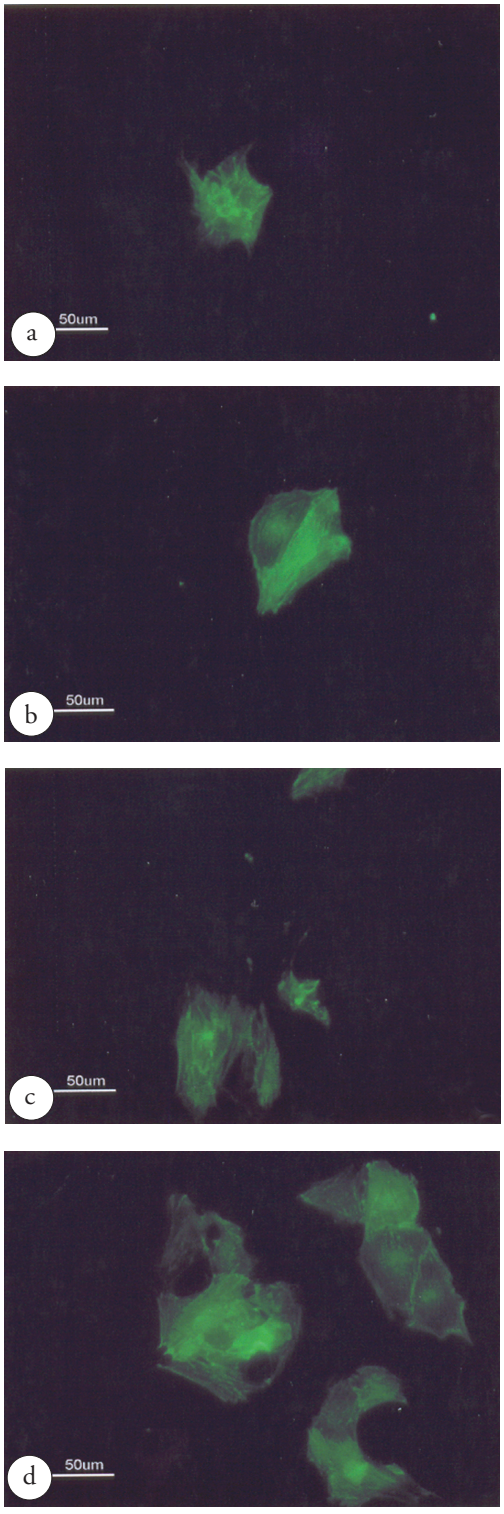

Figure 2 - Immunostaining of embryonic stem cells-derived cardiomyocytes with anti α-actinin. (**a**) and (**b**) indicate control and basic fibroblast growth factor (bFGF) groups in early stage of development (7+3 days), (**c**) and (**d**) indicate control and bFGF groups in late stage of development (7+14 days).

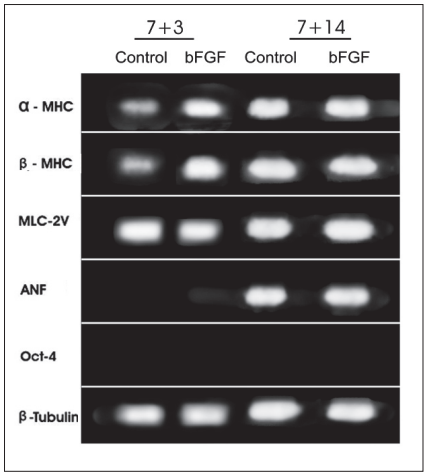

Figure 3 - Expression of cardiac-specific genes at (7+3 days) and (7+14 days) days in control and basic fibroblast growth factor (bFGF) groups analyzed by reaction time polymerase chain reaction. α-MHC - alpha-myosin heavy chain, β–MHC - cardiac beta-myosin heavy chain, MLC-2V - myosin light chain isoform 2V, ANF - atrial natriuretic factor.

presence of functional ion channel in the ESC-derived cardiomyocytes. Both isoprenaline and phenylephrine increased the beating frequency of the cardiomyocytes, but the changes of pre- and post-drug administration were only enhanced in the experimental group at the early stage of development ($p<0.05$ and $p<0.01$). On the other hand, carbachol reduced the beating frequency of the cardiomyocytes at the late stage in the bFGF group ($p<0.05$). The ESC-derived cardiomyocytes of both groups were α-actinin positive, and they showed similar sarcomeric organizations at the early and late stages of development (**Figure 2**).

The isolated ESC-derived cardiomyocytes at the early and late stages of development also expressed cardiac α-MHC, cardiac β-MHC, MLC-2V and β-tubulin in both groups. Nevertheless, the expression of the 2 former genes was more pronounced at the early stage in the bFGF group. The expression of Oct-4 was not detected in both groups after differentiation, whereas the ANF gene was only expressed at the late stage in both groups (**Figure 3**).

Discussion. An the in vitro culture system for the generation of functional cardiomyocytes from ESCs has several potential applications, for example in the fields of developmental biology, transplantation medicine, experimental pharmacology and teratology, the effects of bFGF on the differentiation of ESCs into beating cardiomyocytes along with the specific expression of multiple cardiac-associated molecular markers and their appropriate response to cardioactive drugs are herein

described. Although the precise changes in the membrane ion channel activity or the composition accompanying development and differentiation remained obscure, the EBs cultured in bFGF exhibited superior spontaneous beating characteristics in response to drug treatment. The cardiomyocytes cultured in bFGF responded better to isoprenaline and phenylephrine, while the rate of beats in response to carbachol reduced more in the cardiomyocytes cultured in bFGF in comparison to the ones grown in the control group.

This may indicate an upregulation of α1 and β1-adrenergic receptors at the early stage (7+3 days) and muscarinic cholinergic receptors at the late stage (7+14 days). The expression of α-MHC and β-MHC in the bFGF group was also enhanced at the early stage. However, the beating rate of the bFGF group was less than that of the control group, which may indicate a change in cardiac-specific channels. Receptor-coupled FGF signaling was shown to regulate cardiac myocyte growth during the tubular stages of cardiogenesis.[19] Myocyte growth, however, became FGF-independent after the second week of embryogenesis.[14] Previous studies have demonstrated that in posterior mesoderm explants, a combination of BMP2 and FGF4 can stimulate heart formation, while neither factor alone can do so.[20-22] The present study showed that bFGF alone could not affect the late development of cardiomyocytes and that its combination with other factor(s) is necessary for affecting an increase in cardiomyocyte differentiation. Factor bFGF is involved in the autoregulatory processes of cardiomyocyte proliferation and differentiation[23] and before the processes of down-regulation of initial stages of the vertebrate cardiac development in vivo.[24]

Variable action potentials in ESC-derived cardiomyocytes at different developmental stages are said to be well correlated with the expression of specialized types of ion channels.[25] In cardiomyocytes of early differentiation stage, the primitive pacemaker-action potentials are generated by only 2 main types of ion channels: voltage dependent L-type Ca^{2+} channels (ICa) and transient K^{+} channels. The increase of response in the cardiomyocytes in our study to the drugs from 7+3 days to 7+14 days could be related to the aforementioned channels. In addition, the effect of sympathomimetics is through Na^{+} and Ca^{2+} pumps.[26] Improvement in these channels together with the development of the cells can be necessary for an increase in their response to these drugs.

The data presented herein suggests that the pluripotent ESCs cultivated via EBs reproduce cardiomyocytic development from primitive precursor cells to highly specialized cardiac cells. In-vitro-differentiated cardiomyocytes seem to resemble the in vivo development of cardiomyocytes and thus, may be ideal for more detailed studies on the commitment and differentiation of cardiomyocytes; the role of growth factors and extracellular matrix components; cardiac myogenesis; and pharmacological and toxicological effects on cardiomyocytic morphology, gene expression, cardiac–specific ionic currents, and action potentials. The ESC-derived cardiomyocytes can be useful for clinical applications. Further in vivo studies, however, are needed to explore the pros and cons of cellular therapies with these cells.

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