

Efficient Cardiomyogenic Differentiation of Embryonic Stem Cell by Fibroblast Growth Factor 2 and Bone Morphogenetic Protein 2

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Background Despite the pluripotency of embryonic stem (ES) cells, the specific control of their cardiomyogenic differentiation remains difficult. The aim of the present study was to investigate whether growth factors may efficiently enhance the in vitro cardiac differentiation of ES cells.

Methods and Results Recombinant growth factors at various concentrations or their inhibitors were added according to various schedules during the cardiomyogenic differentiation of ES cells. Cardiomyogenic differentiation was assessed by mRNA and protein expressions of several cardiomyocyte-specific genes. Basic fibroblast growth factor-2 (FGF-2) and/or bone morphogenetic protein-2 (BMP-2) efficiently enhanced the cardiomyogenic differentiation, but only when they were added at the optimal concentration (1.0 ng/ml in FGF-2 and 0.2 ng/ml in BMP-2; relatively lower than expected in both cases) for the first 3 days. Inhibition of FGF-2 and/or BMP-2 drastically suppressed the cardiomyogenic differentiation.

Conclusion FGF-2 and BMP-2 play a crucial role in early cardiomyogenesis. The achievement of efficient cardiac differentiation using both growth factors may facilitate ES cell-derived cell therapy for heart diseases as well as contribute to developmental studies of the heart. (Circ J 2004; 68: 691–702)

Key Words: Cardiomyogenic differentiation; Embryonic stem cell; Gene expression; Growth factors

The pluripotency of embryonic stem (ES) cells has great potential for facilitating ES cell therapy for some heart diseases, as well as for elucidating the developmental mechanisms of the heart.^{1,2} Because of the lack of proliferative and regenerative activity of differentiated cardiomyocytes after birth, many heart diseases, such as myocardial infarction and cardiomyopathy, are irreversible and incurable by current treatments. One of the experimentally promising strategies is transplantation of cardiomyocytes; recent animal studies have shown that transplantation of fetal or neonatal cardiomyocytes not only results in successful integration into the recipient heart but also apparently improves heart disorders.^{1–6} However, the clinical application of such cell transplantation therapy is completely hampered by the lack of an available source of human cardiomyocytes. In this regard, ES cell-derived cardiomyocytes are a potential candidate for the donor cells. On the other hand, it is still difficult to control and induce the specific differentiation of ES cells solely towards cardiomyocytes, although random differentiation of ES cells leads to the appearance of cells that possess features

of cardiomyocytes to some degree.⁷

Certain growth factors can induce specific types of cells from ES cells.^{8,9} For example, interleukin (IL)-3 directs ES cells to become macrophages, mast cells or neutrophils;¹⁰ IL-6, retinoic acid and transforming growth factor (TGF)- β 1 respectively induced erythroid differentiation, neuronal formation and myogenesis of ES cells.^{11–13} To date, however, there has been no report that growth factors efficiently and specifically induced mouse ES cells to become cardiomyocytes except for one recent report, which investigated TGF- β and bone morphogenetic protein (BMP)-2 only, not fibroblast growth factor (FGF)-2.¹⁴ Developmental studies using knockout mice or chicken embryos have demonstrated that certain growth factors, especially TGF- β ,¹⁵ activin,¹⁶ FGF-2^{17,18} and BMP-2^{19–23} may each play a role in heart development. In the present study, we initially screened these 4 growth factors, and, based on the results, focussed particularly on and carefully explored the effects of FGF-2 and BMP-2 on the cardiomyogenic differentiation of ES cells. Thus we elucidated several roles of FGF-2 and BMP-2 in cardiac development, and for the first time established a system of efficient cardiomyogenic differentiation of ES cells using FGF-2 and BMP-2.

Methods

Cell Cultures

Murine R1 ES cells were grown in an undifferentiated state on mitomycin C-treated mouse embryonic fibroblasts with high glucose Dulbecco's Modified Eagle's Medium (DMEM) with 20% fetal calf serum (FCS), 100 μ mol/L 2-mercaptoethanol (2-ME), 1 μ mol/L sodium pyruvate, 0.1 μ mol/L nonessential amino acids and 10³ unit/ml leuke-

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Table 1 PCR Primers

	Sense	Antisense	Annealing temperature (°C)	Reference
<i>Nkx2.5</i>	5'-CAGTGGAGCTGGACAAAGCC-3'	5'-TAGCGACGGTCTGGAACCA-3'	58	29
<i>GATA4</i>	5'-CTGTCATCTCACTATGGGCA-3'	5'-CCAAGTCCGAGCAGGAATT-3'	58	29
<i>MEF2C</i>	5'-AGCAAGAATACGATGCCATC-3'	5'-GAAGGGTGGTGGTACGGTC-3'	58	29
<i>αMHC</i>	5'-GGAAGAGTGAGCGGCCATCAAGG-3'	5'-CTGCTGGAGGTTATCTCG-3'	58	29
<i>FGFR-1</i>	5'-GCTGACTCTGGCCTCTACGCT-3'	5'-CAGGATCTGGACATACGGCAA-3'	62	17
<i>FGFR-2</i>	5'-CTCCTTCAGTTTGTGAGGATACCA-3'	5'-GAAGATCCAAGTTTCACTGTC TACCG-3'	60	17
<i>FGFR-3</i>	5'-GAAGAATGGCAAAGAATTCCGAG-3'	5'-CCTCTAGCTCCTTGTCGGTGG-3'	60	17
<i>FGFR-4</i>	5'-GAACTCTCTGGGTAGCATTGCT-3'	5'-TGTCTGTTGCTTGAGGAC TTGTACG-3'	58	17
<i>BMPR-1A</i>	5'-TCGTCTGTATTACAGGAG-3'	5'-TTACATCTGGGATTCAACC-3'	58	30
<i>BMPR-1B</i>	5'-GCTTTGGACTCATCCTCTGG-3'	5'-CACTGGGCAGTAGGCTAACG-3'	54	30
<i>ActR-1</i>	5'-AGATGACGTGAAGACCCCG-3'	5'-ATACTTCTCCATAGCGGCC-3'	56	30
<i>BMPR-2</i>	5'-GGTAGATAGAGGGAACGGC-3'	5'-CACTGCCATTGTTGTTGACC-3'	56	30
<i>HPRT</i>	5'-CCTGCTGGATTACATTAAAGCACTG-3'	5'-AAGGGCATATCCAACAACA-3'	58	

MEF2C, myocyte enhancer factor 2C; *αMHC*, amylin heavy chain; *FGFR*, fibroblast growth factor receptor; *BMPR*, bone morphogenetic protein receptor; *ActR-1*, activin receptor-1; *HPRT*, hypoxanthine-phosphoribosyl-transferase.

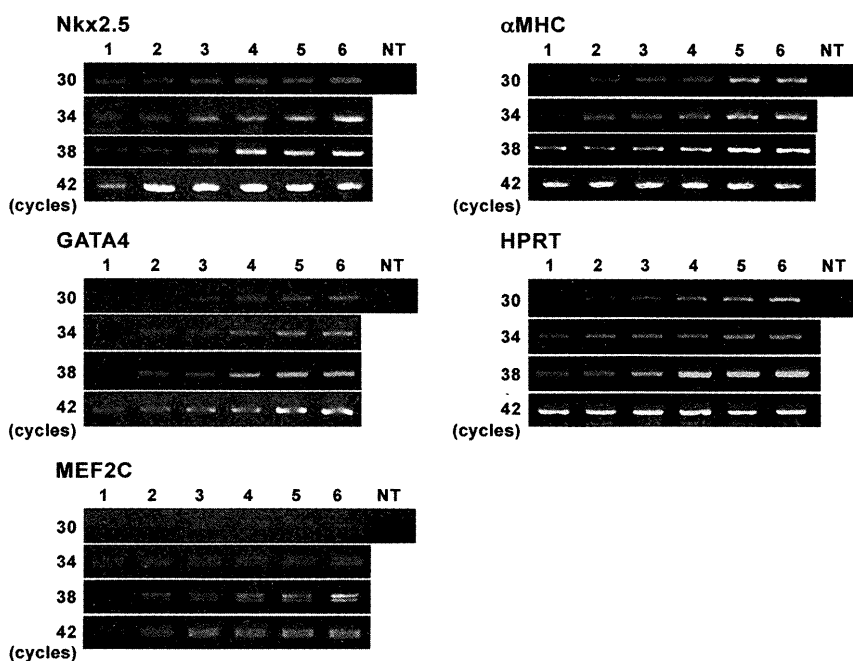


Fig 1. Optimization of the semi-quantitative RT-PCR assay. Serially diluted plasmid DNA containing *Nkx2.5* cDNA (0.08, 0.15, 0.31, 0.63, 1.25 and 2.5 pg DNA in lanes 1, 2, 3, 4, 5 and 6, respectively) was used for control samples for semi-quantitative RT-PCR of *Nkx2.5*. Total RNA extracted from adult mouse heart was serially diluted (1.3, 2.5, 5, 10, 20 and 40 ng RNA in lanes 1, 2, 3, 4, 5 and 6, respectively), reverse-transcribed and used for control samples for semi-quantitative RT-PCR of *GATA4*, *MEF2C*, *αMHC* and *HPRT*. PCR was carried out for 30, 34, 38 and 42 cycles using each of the primer sets shown in Table 1. The amplified cDNA was electrophoresed onto 2% agarose gel containing ethidium bromide.

mia inhibitory factor (LIF)^{24–26} To initiate the differentiation, 10^6 ES cells were cultured with DMEM containing 10% FCS and 100 μ mol/L 2-ME, but no LIF, in 10 cm low-attachment Petri dishes to generate embryoid bodies (EBs). After 3 days in suspension, the EBs were transferred into gelatin-coated 12-well tissue culture dishes at a density of 10–20 EBs per 3.5 cm², and cultured for an additional 7 or 14 days. Recombinant growth factors at various concentrations or their inhibitors were added to the culture media according to different schedules, as described in the Results section. The recombinant growth factors (FGF-2, BMP-2, TGF- β and activin A) and their inhibitors (anti-FGF-2 antibody, BMPR-1B/Fc chimera²⁷ Noggin/Fc chimera^{19,28} anti-TGF- β antibody and anti-platelet-derived growth factor (PDGF) antibody) were all purchased from R&D systems (Minneapolis, MN, USA).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from differentiated ES cells on days 3+7 and 3+14 using a Sepazol RNA 1 super kit

(NACALAI TESQUE, Inc, Kyoto, Japan) according to the manufacturer's protocol. For semi-quantitative RT-PCR analysis, 1 μ g of total RNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen Corp, Carlsbad, CA, USA), and then 1/100 of the cDNA was subjected to PCR amplification by 30–42 cycles of 94°C for 30s, each annealing temperature for 90s and 72°C for 60s using each of the primer sets shown in Table 1^{17,29,30} The amplified cDNA was electrophoresed on 2% agarose gel containing ethidium bromide and the quantities were analyzed by densitometry with NIH IMAGE software (the Research Service Branch of the National Institute of Health, Bethesda, MD, USA). The most appropriate PCR cycles for the semi-quantitative analysis for each experiment were carefully determined by several preliminary experiments; somewhat less (30–35) or more (38) cycles were suitable for accurately assessing the inducible or the inhibitory effects, respectively (Fig 1). Moreover, *Nkx2.5*/HPRT or *αMHC*/HPRT ratio relative to that of NC (non-treatment control) was calculated for facilitating comparison of the relative effects on different conditions in the individual

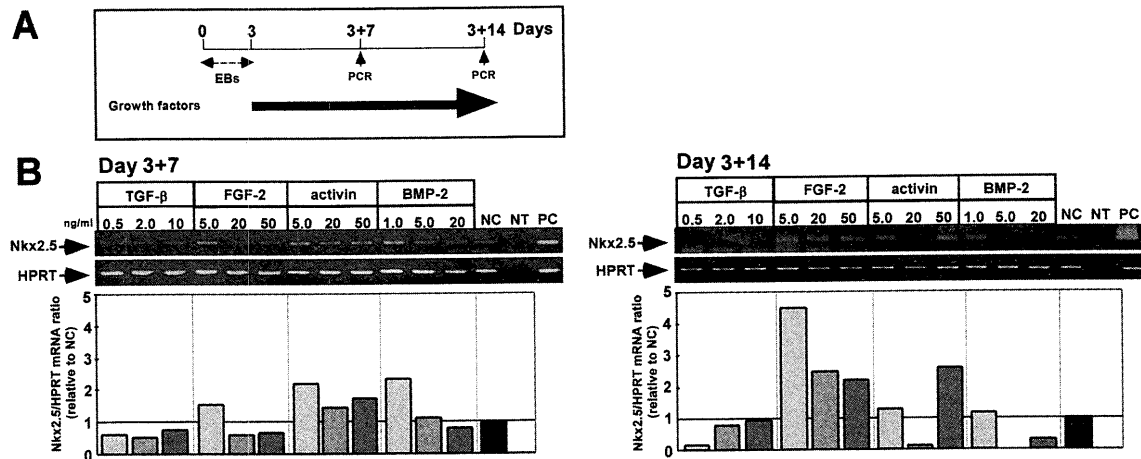


Fig 2. Nkx2.5 mRNA expression in ES cells treated with one of the 4 growth factors. (A) Experimental schedule. ES cells were cultured in suspension for 3 days to form EBs, and subsequently plated and cultured with media containing one of 4 recombinant growth factors at various concentrations for an additional 7 or 14 days. (B) Expression of Nkx2.5 mRNA was detected by RT-PCR. The Nkx2.5/HPRT mRNA ratio in each treatment group was standardized by and expressed as the relative ratio to that of the non-treatment control (NC; ES cells that were cultured without the addition of recombinant growth factors). PC, positive control (adult mouse heart tissue); NT, RT-PCR with no template; Nkx2.5/HPRT mRNA ratio in each treatment group was standardized by and expressed as the relative ratio to that of NC.

experiments, as well as standardizing the unavoidable variability of PCR data and cell conditions in them. The reproducibility of all the results was confirmed by at least 3 independent experiments.

Immunocytochemistry and Computer-Assisted Morphometric Analysis

Differentiated ES cells on days 3+7 or 3+14 were fixed in 4% paraformaldehyde for 45 min and permeabilized with 100% ethanol for 2 min. For immunofluorescent staining, cells were incubated with a primary antibody of anti- α -actinin (Sigma-Aldrich Inc, St Louis, MO, USA) or anti-Nkx2.5 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at room temperature for 1 h. After washing, cells were incubated with a secondary antibody of donkey anti-mouse Alexa 488 or Alexa 568 antibodies (Molecular Probes Inc, Eugene, OR, USA) at room temperature for 1 h.

For quantitative analysis of the percentage of ES cell-derived cardiomyocytes that express cardiomyocyte-specific sarcomeric proteins, cells were stained by the immuno-peroxidase method using a LSAB2 kit (DAKO CORPORATION, Carpinteria, CA, USA) according to the manufacturer's protocol. Briefly, cells were incubated with a primary monoclonal antibody of MF-20 that recognizes sarcomeric myosin (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA)^{29,31} or anti-sarcomeric tropomyosin (Sigma-Aldrich Inc) for 1 h at room temperature. After washing, cells were incubated with a secondary antibody of biotinylated rabbit anti-goat IgG for 1 h at room temperature, and subsequently with streptavidin for 10 min and with 3,3'-diaminobenzidine tetrahydrochloride for 20 min. To accurately quantify the percentage of positive cells, computer-assisted morphometric analysis was performed using Adobe Photoshop 7.0 software (Adobe Systems Inc, San Jose, CA, USA) as follows. More than 20 fields of immunohistochemically-stained specimens at a magnification of $\times 100$ (comprising a total $>10^4$ cells) were chosen at random and scanned, and then the positive and negative signals were transferred to digital images. The percentage of the total area showing positive

signals was automatically calculated. To quantify the Nkx2.5-expressing cells, the number of Nkx2.5-positive nuclei by immunofluorescent staining in the measured area was counted using computer-assisted morphometric analysis in the same manner. All these analyses were done strictly as double-blind tests, and the reproducibility of all the results was confirmed by at least 3 independent experiments.

Statistical Analysis

All data are represented as the mean \pm standard deviation. Statistical significance was evaluated using Student's t-test for unpaired comparison, and values of $p < 0.05$ were considered to indicate statistical significance.

Results

Screening of the 4 Growth Factors for Efficient Cardiomyogenic Differentiation of ES Cells

Accumulating data in developmental studies have suggested that TGF- β , FGF-2, activin, and BMP-2 play particularly important roles in the development and differentiation of the heart. First, we did an initial screening of the potential activities of these 4 growth factors to enhance in vitro cardiomyogenic differentiation of ES cells by determining the expression levels of Nkx2.5 mRNA. Nkx2.5 is a mouse homeobox gene, a cardiomyocyte-specific transcriptional factor and one of the earliest genes expressed in the heart during its development.³² Nkx2.5 may regulate multiple genes essential for heart development and is expressed in the heart even after birth.³³ Our kinetic data showed that expression of Nkx2.5 mRNA was detected at a faint level as early as day 3+0 and was stable between day 3+2 and day 3+14 (data not shown). Therefore, we chose Nkx2.5 for the initial screening; each of the 4 growth factors was added to the culture media at diverse concentrations between day 3+1 and day 3+14 and the mRNA levels of Nkx2.5 were examined on day 3+7 and day 3+14 (Fig 2A). An apparent increase in Nkx2.5 mRNA expression was seen on day 3+7 when FGF-2 or BMP-2 was

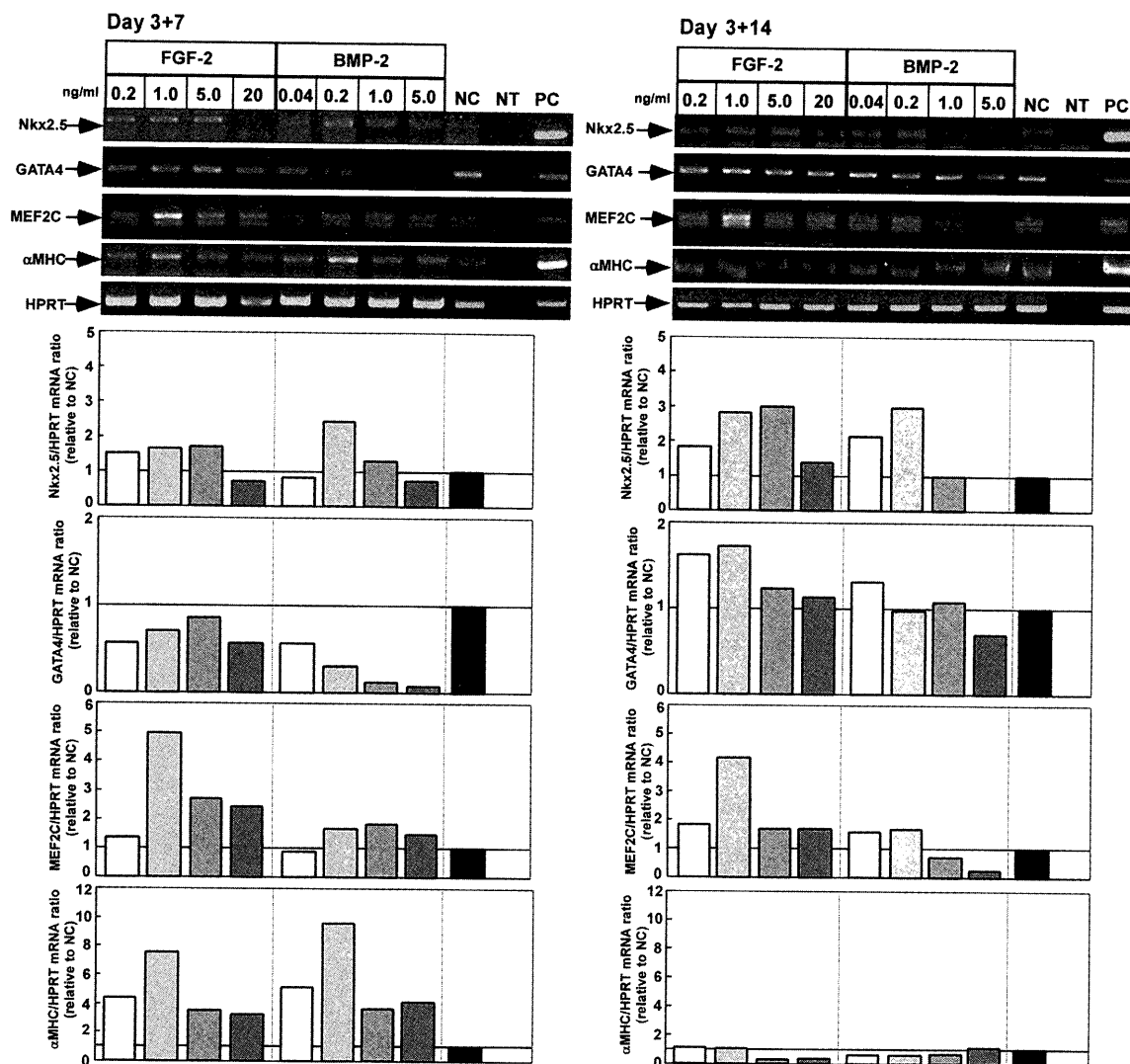


Fig 3. Effects of various doses of FGF-2 or BMP-2 on in vitro cardiomyogenic differentiation of ES cells. ES cells were cultured with FGF-2 or BMP-2 at various concentrations on the same schedule as shown in Fig 2A. RT-PCR analysis of cardiomyocyte-specific genes, Nkx2.5, GATA4, MEF2C and α MHC, was performed. The same results were obtained in 3 independent experiments. The Nkx2.5/HPRT, GATA4/HPRT, MEF2C/HPRT or α MHC/HPRT mRNA ratio in each treatment group was standardized by and expressed as the relative ratio to that of the non-treatment control (NC).

added at a final concentration of 5.0 or 1.0 ng/ml, respectively (Fig 2B). Unexpectedly, the expression levels of Nkx2.5 were actually decreased when FGF-2 or BMP-2 was used at concentrations higher than 5.0 or 1.0 ng/ml, respectively. On the other hand, the expression levels of Nkx2.5 mRNA were not significantly changed by the addition of TGF- β . Activin enhanced Nkx2.5 expression to some degrees in comparison to the non-treatment control (NC), but the changes in the Nkx2.5 expression levels did not show a dose-related or consistent pattern between day 3+7 and day 3+14.

Relatively Low Concentrations of FGF-2 or BMP-2 Effectively Enhanced the Cardiomyogenic Differentiation of ES Cells

Based on the results from the initial screening experiment, we decided to focus on FGF-2 and BMP-2 in the present study; in particular, further investigation of FGF-2 was thought to be important because of the lack of previous reports, as well as the striking increases in Nkx2.5 mRNA

on day 3+14 in the initial screening (Fig 2). To determine the optimal concentration of FGF-2 or BMP-2 for efficient cardiomyogenic differentiation, a lower concentration of FGF-2 or BMP-2 was added according to the same schedule, and the expression levels of Nkx2.5, GATA4, MEF2C and α MHC mRNA were explored by RT-PCR analysis (Fig 3). GATA4 is expressed in the adult vertebrate heart, as well as in yolk sac endoderm and cells involved in heart formation.³⁴ The murine MEF2C is expressed in heart precursor cells before formation of the linear heart tube.³⁵ α MHC is one of the representative sarcomeric proteins and thus is expressed in mature and differentiated cardiomyocytes.³⁶ In fact, expression of α MHC mRNA was detected in ES cells at a faint level as early as day 3+5 and stable between day 3+8 and day 3+14 (data not shown). Thus, Nkx2.5, GATA4, MEF2C and α MHC mRNA were suitable markers for cardiomyogenic differentiation at various stages.

Significant increases in the expression of Nkx2.5 were seen on day 3+7 and day 3+14 when FGF-2 was added at

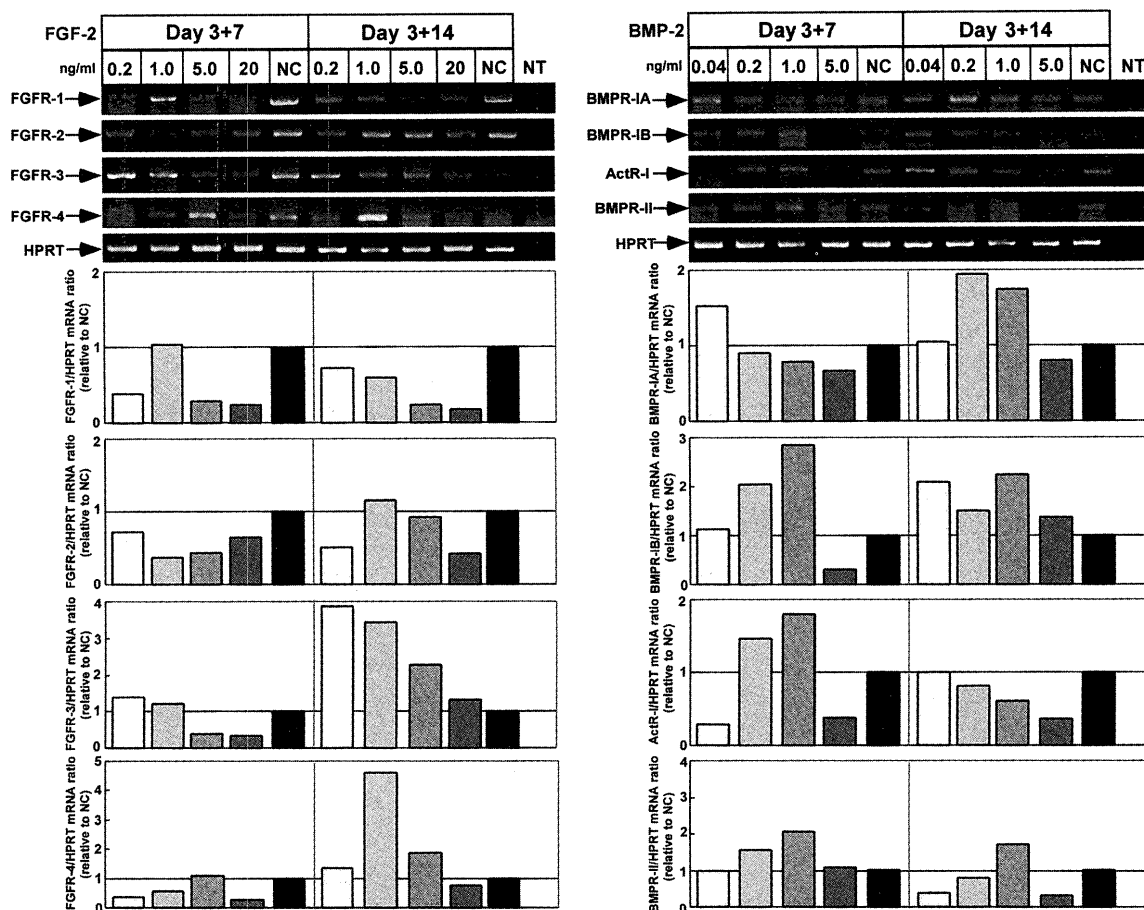


Fig4. Effects of various doses of FGF-2 or BMP-2 on the expression levels of FGFRs and BMPRs in ES cells. Experimental schedule was the same as shown in Fig 2A. RT-PCR analyses of FGFRs (FGFR-1, FGFR-2, FGFR-3 and FGFR-4) and BMPRs (BMPR-IA, BMPR-IB, ActR-I and BMPR-II), and the calculation of FGFR/HPRT or BMPR/HPRT mRNA ratio were performed in the same way as shown in Fig 3. The same results were obtained in 3 independent experiments.

a final concentration of 1.0–5.0 ng/ml. The expression of α MHC on day 3+7 and of MEF2C on day 3+7 and day 3+14 was apparently increased by the addition of FGF-2 within this range (0.2–20 ng/ml); the most striking increase was seen in the case of 1.0 ng/ml FGF-2. The expression of GATA4 was slightly increased on day 3+14 by additions of FGF-2 at a lower concentration (0.2–1.0 ng/ml) despite a slight decrease on day 3+7. On the other hand, the most striking increase in the expression of Nkx2.5 on day 3+7 and day 3+14 and of α MHC on day 3+7 was seen only when BMP-2 was added at a final concentration of 0.2 ng/ml. The expression of MEF2C slightly increased on day 3+7 and day 3+14 after the addition of BMP-2 at the concentration of 0.2 ng/ml. Interestingly, the expression of GATA4 was remarkably inhibited on day 3+7 by the addition of BMP-2 in a dose-dependent manner, although the expression normalized or slightly increased at lower concentrations (0.04–1.0 ng/ml). Thus, the optimal concentrations of FGF-2 and BMP-2 are 1.0 and 0.2 ng/ml, respectively; both of which are relatively low (ie, lower than expected from previous developmental studies) and the effective range of each growth factor for cardiomyogenic differentiation is thus also relatively narrow.

On the other hand, the expression of α MHC on day 3+14 did not in appearance show positive findings for inducible effects in comparison with the NC. It should be noted that

all the PCR data, including those shown in Fig 3, represent the ratio relative to the NC in each experiment, and that the actual expression level of α MHC in the NC was higher on day 3+14 than on day 3+7 (Fig 3). In fact, some clusters of ES cells in all groups, including the NC, demonstrated a contraction between day 3+5 and day 3+14, and the number of contracting clusters of ES cells was increasing later and most prominently around day 3+10. Its number on day 3+7 was somewhat increased when the optimal concentration of FGF-2 or BMP-2 was added; however, there was no apparent difference in this number among all groups on day 3+14, and the number of contracting clusters of ES cells was somewhat decreased on day 3+14 in some cases (data not shown). Hypothetically, certain inhibitory molecules against later cardiomyogenic differentiation might be endogenously expressed in a negative feed-back manner under such matured conditions; in fact, ES cells even in the NC were fully grown in a confluent condition on day 3+14. Up-regulation of Nkx2.5 on day 3+14 as well as day 3+7 further suggests that both growth factors may play a more important role in early cardiomyogenic differentiation than later.

It is known that the major FGF receptor (FGFR) in the heart is FGFR-1, whereas the biological roles of other FGFRs (ie, FGFR-2, FGFR-3 and FGFR-4) in the heart remain unknown.^{17,37} The expression of FGFR-1 decreased

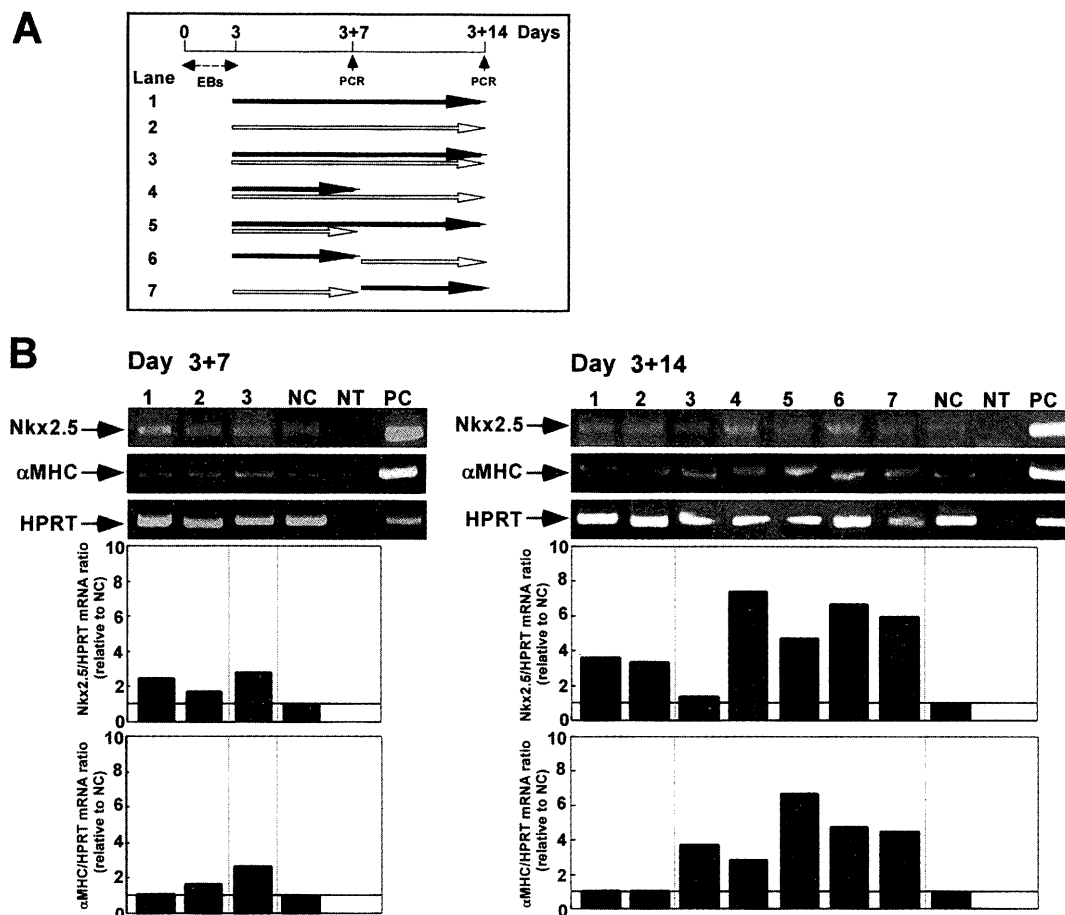


Fig 5. Effects of the combination of FGF-2 and BMP-2 on in vitro cardiomyogenic differentiation of ES cells. (A) Experimental schedules. ES cells were cultured with FGF-2 (1.0 ng/ml) and BMP-2 (0.2 ng/ml) following different schedules. Black and white arrows represent the addition of FGF-2 and BMP-2, respectively. (B) RT-PCR analysis of Nkx2.5 and α MHC was done and the Nkx2.5/HPRT or α MHC/HPRT mRNA ratio was calculated in the same way as shown in Fig 3.

on both day 3+7 and day 3+14 after addition of FGF-2 at higher concentrations, whereas those of FGFR-3 and FGFR-4 were remarkably increased on day 3+14 after addition of FGF-2 at lower concentrations (Fig 4). On the other hand, 3 structurally related type I receptors, BMP receptor (BMPR) I (BMPR-IA, BMPR-IB) and activin receptor I (ActR-I) and 1 type II receptor, BMPR-II, have been identified as the specific receptors for BMP. However, the overall role of these BMPRs in the cardiac development remains unknown except for the fact that all BMPRs are expressed in cultured neonatal rat cardiomyocytes³⁸. Addition of BMP-2 at lower (certain) or higher concentrations showed a general tendency to increase or decrease the expressions of BMPRs on day 3+7 and day 3+14, respectively (Fig 4). Thus, the addition of FGF-2 or BMP-2 at higher concentrations may lead to down-regulation of their receptors, implying a possible relationship with their reduced effects for cardiomyogenic differentiation.

Combination of FGF-2 and BMP-2 on Diverse Schedules

FGF-2 and BMP-2 may coordinate their functions in cardiomyogenic differentiation during the development of the heart. To investigate whether the combination of FGF-2 and BMP-2 might further enhance the in vitro cardiomyogenic differentiation of ES cells, we added both FGF-2 and BMP-2 on diverse schedules, at the optimal concentrations (1.0 ng/ml in FGF-2 and 0.2 ng/ml in BMP-2) determined

by the previous experiment (Fig 5A). In comparison to FGF-2 or BMP-2 alone, further increases in Nkx2.5 and α MHC mRNA expression on day 3+7 and day 3+14 were seen in all groups with the combination of both growth factors (Fig 5B). There were no significant differences or specific patterns in the Nkx2.5 or α MHC mRNA levels on day 3+14 among the combination groups (lanes 4–7 in Fig 5B) except for the finding of a smaller increase in Nkx2.5 in the case of the addition of both growth factors on each of the 14 days (lane 4 in Fig 5B). The degree of further up-regulation of Nkx2.5 on day 3+7 and day 3+14 and of α MHC on day 3+7 (ie, the ratio of the mRNA levels in these combination groups (except lane 4 in Fig 5B) relative to those in the group having FGF-2 or BMP-2 alone) was roughly 1.5–2-fold. Thus, FGF-2 and BMP-2 may independently and additively up-regulate both transcription of Nkx2.5 and cardiomyogenic differentiation, although the timing of the addition of FGF-2 and/or BMP-2 after the formation of EBs was not the definitive factor for their effectiveness.

BMP-2 and FGF-2 Play Important Roles in the Early Cardiomyogenic Differentiation of ES Cells

Recent developmental studies have shown that both BMP-2 and FGF-2 play an important role in early heart development, especially in the induction of the mesoderm component at the time of the formation of the 3 germ

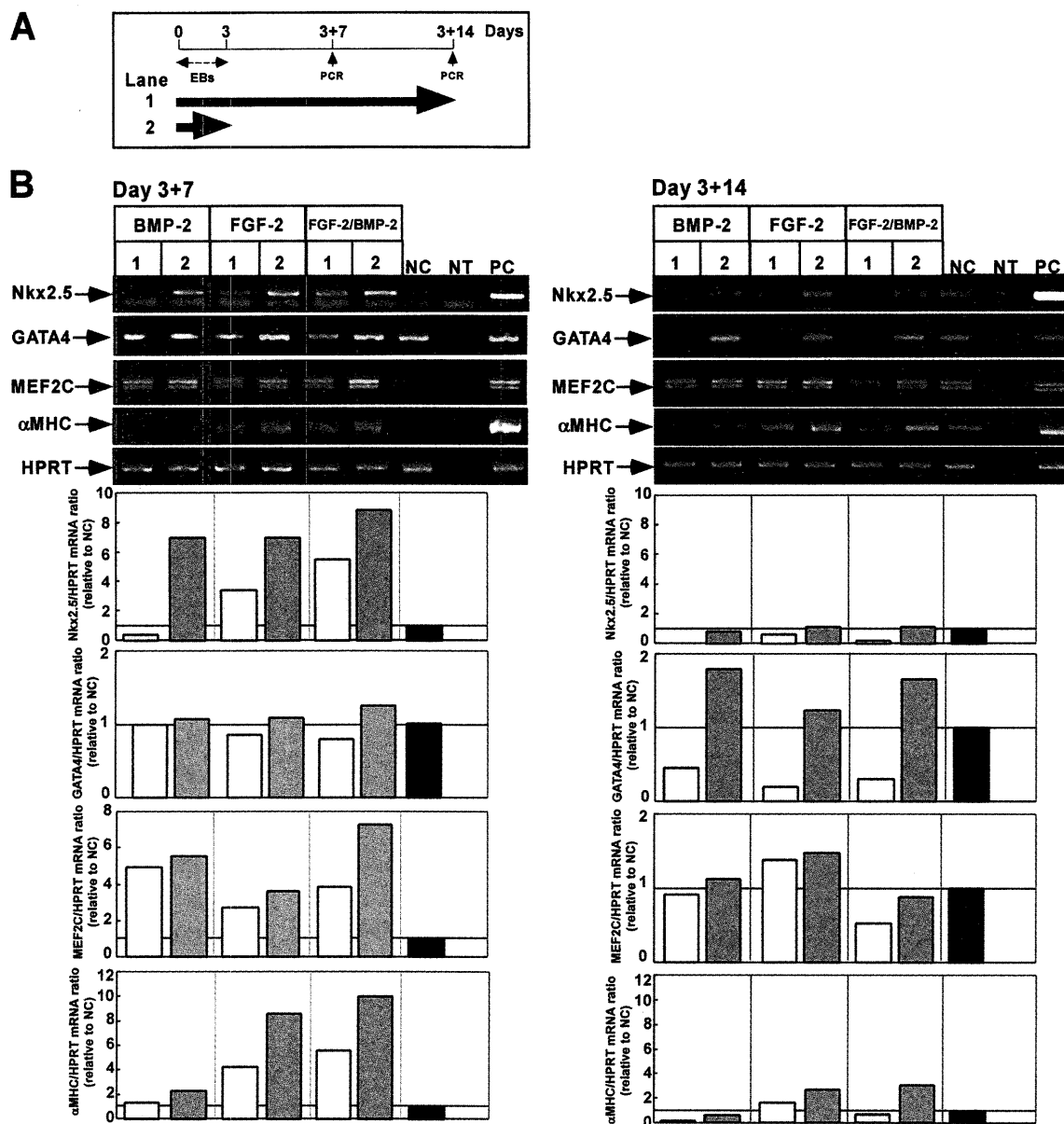


Fig 6. Efficacy of FGF-2 and BMP-2 for early cardiomyogenic differentiation. (A) Experimental schedules. In vitro differentiation was initiated by the formation of EBs without LIF in the same way as already shown, and FGF-2 (1.0 ng/ml) and/or BMP-2 (0.2 ng/ml) were added to the culture media either during the whole 17 days (lane 1) or during only the first 3 days (lane 2). (B) RT-PCR analysis of Nkx2.5, GATA4, MEF2C and α MHC and the calculation of the Nkx2.5/HPRT, GATA4/HPRT, MEF2C/HPRT or α MHC/HPRT mRNA ratio were performed in the same way as shown in Figs 3 and 5. Lanes 1 and 2 correspond to those shown in Fig 6A.

layers. In the present experimental schedule, this stage may have corresponded to the first 3 days, when the EBs were being formed. To explore the roles of both growth factors in early cardiomyogenic differentiation, FGF-2 and/or BMP-2 were added either on each of the 17 days, or only on the first 3 days, and the efficiency of the cardiomyogenic differentiation was compared between these 2 schedules (Fig 6A). Interestingly, the expression levels of Nkx2.5, GATA4, MEF2C and α MHC on both day 3+7 and day 3+14 were significantly higher after the addition of either or both growth factors in the group with the 3-day schedule than in the group with the 17-day schedule (Fig 6B). The best outcomes in all the parameters (ie, expression levels of Nkx2.5, GATA4, MEF2C and α MHC mRNA on day 3+7 and day 3+14) were obtained by the addition of both

growth factors with the 3-day schedule. Interestingly, the effects of FGF-2 for up-regulation of α MHC were somewhat more prominent than those of BMP-2 in the case of this experimental protocol with the 3-day schedule; this finding was not apparent in the previous experimental schedules (ie, addition of the growth factors only after the formation of EBs), as shown in Figs 1–3. The drastic increases in Nkx2.5, GATA4, MEF2C and α MHC mRNA in the group with the 3-day schedule of either FGF-2 or both growth factors clearly indicates that BMP-2 and FGF-2 are crucial in the early stage of cardiomyogenic differentiation of ES cells, but not throughout all the stages. It should be noted that the addition of BMP-2 at later stages in addition to the early stage may actually exhibit an inhibitory effect for the cardiomyogenic differentiation.

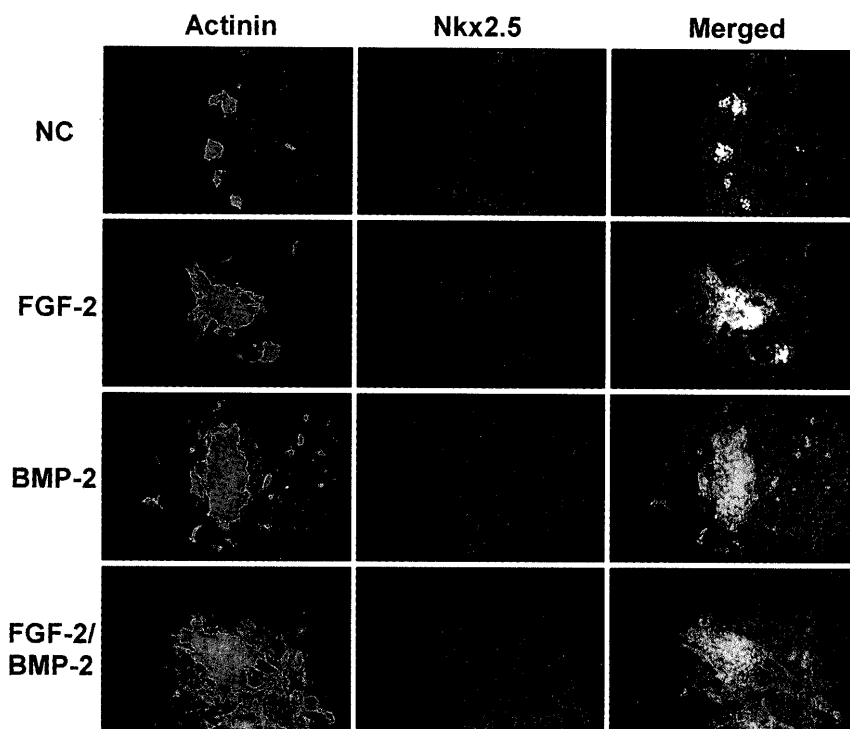


Fig 7. Immunocytochemical staining of cardiomyocyte-specific genes, actinin and Nkx2.5. FGF-2 (1.0 ng/ml) and/or BMP-2 (0.2 ng/ml) were added for 7 days after the formation of EBs using the same protocol as shown in Fig 5A. ES cells on day 3+7 were immunocytochemically stained with anti-actinin and anti-Nkx2.5 antibodies. NC, non-treatment control (Original magnification, $\times 100$).

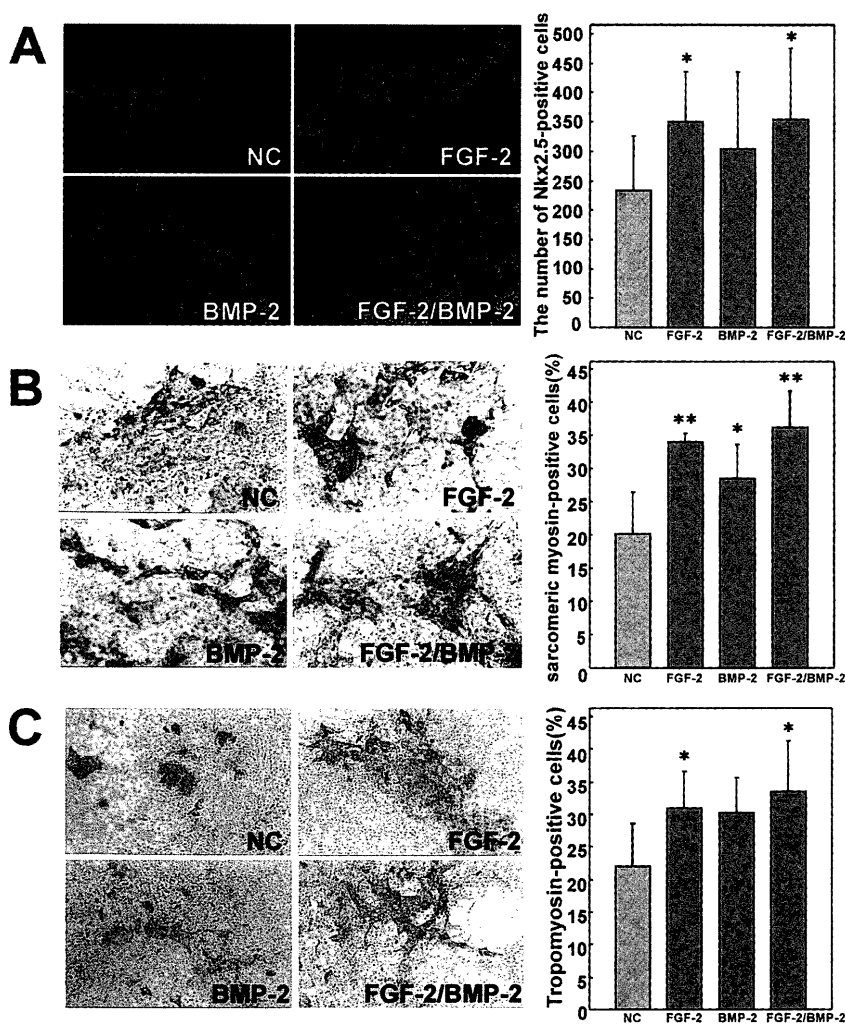


Fig 8. Immunohistochemical and quantitative analysis of ES cell-derived cardiomyocytes. ES cells were cultured with FGF-2 (1.0 ng/ml) and/or BMP-2 (0.2 ng/ml) under the same protocol as shown in Fig 5A. ES cells on day 3+7 were immunocytochemically stained with an antibody of anti-Nkx2.5 (A), anti-sarcomeric myosin (B) or anti-tropomyosin (C). Computer-assisted morphometric analysis was performed. NC, non-treatment control (Original magnification of all pictures of immunocytochemically-stained specimens, $\times 100$). * $p < 0.05$; ** $p < 0.001$.

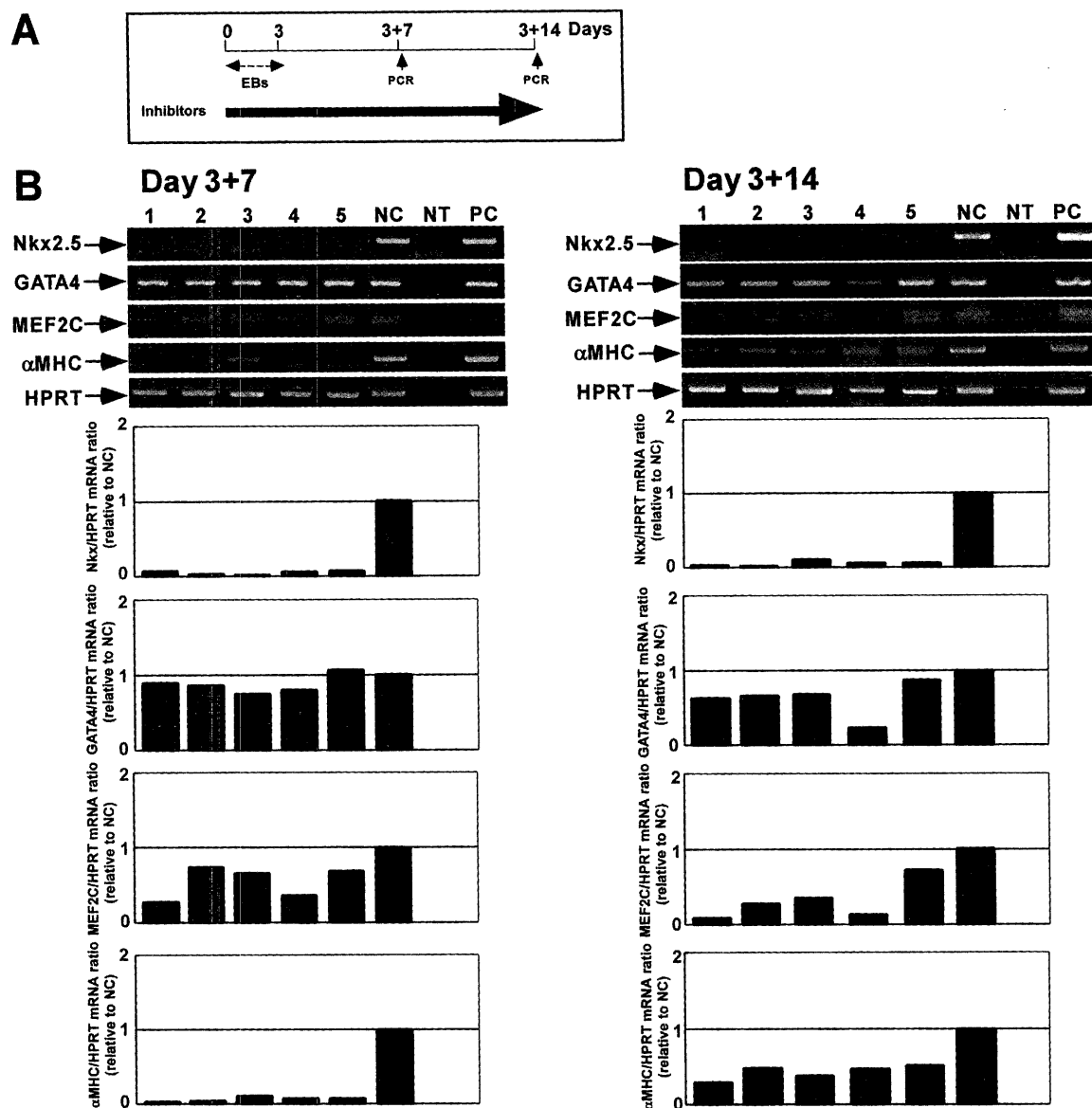


Fig 9. Inhibition of FGF-2 and BMP-2 attenuated in vitro cardiomyogenic differentiation of ES cells. (A) Experimental schedule. In vitro differentiation was initiated by the formation of EBs without LIF in the same way as already shown. Either anti-FGF-2 antibody (2.5 μ g/ml; lane 1), BMPR-1B/Fc (1.0 μ g/ml; lane 2), Noggin/Fc chimera (0.1 μ g/ml; lane 3), anti-TGF- β antibody (1.5 μ g/ml; lane 4) or anti-PDGF antibody (1.5 μ g/ml; lane 5) were added during the whole 3+7 or 3+14 days. (B) RT-PCR analysis of Nkx2.5, GATA4, MEF2C and α MHC, and the calculation of the Nkx2.5/HPRT, GATA4/HPRT, MEF2C/HPRT or α MHC/HPRT, mRNA ratio were performed in the same way as shown in Figs 3 and 5.

Quantifying ES Cell-Derived Cardiomyocytes by Immunocytochemistry

To confirm the cardiomyogenic differentiation of ES cells, ES cell clusters were immunocytochemically stained with anti-actinin, one of the representative cardiomyocyte-specific sarcomeric proteins, together with anti-Nkx2.5 antibodies (Fig 7). Contracting clusters of ES cells on day 3+7 were specifically stained with both antibodies, suggesting that the majority of the contracting ES cells possessed the features of cardiomyocytes. Addition of growth factors at the optimal concentrations (1.0 ng/ml in FGF-2 and 0.2 ng/ml in BMP-2) on the optimal schedule (ie, during the first 3 days only; (Fig 6A)), increased the number of ES cells that expressed both of the cardiomyocyte-specific proteins, in accordance with the previous results of the mRNA levels by RT-PCR (Fig 6B).

Moreover, we quantified the percentage of ES cell-derived cardiomyocytes by computer-assisted morphometric analyses of Nkx2.5-, sarcomeric myosin- or tropomyosin-positive cells (Fig 8). The results showed that each of FGF-2 alone, BMP-2 alone, and the combination treatment with both growth factors significantly enhanced the cardiomyogenic differentiation, and FGF-2 had a somewhat more prominent effect on the cardiomyogenic differentiation than BMP-2, in accordance with the previous results of mRNA levels (Fig 6B). The best result was obtained when both FGF-2 and BMP-2 were added; finally, sarcomeric myosin-positive cells made up approximately 35% of all ES cells and 1.5-fold as many as in the NC (approximately 20% positive cells).

Inhibition of FGF-2 and/or BMP-2 Attenuated the Cardiomyogenic Differentiation

The formation of EBs without LIF in the culture media induces the cardiomyogenic differentiation of ES cells to some degree, even when nothing is added, and this is so far the only available procedure to prime this event. A hypothesis is that FGF-2 and/or BMP-2 may be endogenously secreted from ES cells and these endogenous factors may prime the event and/or regulate the cardiomyogenic differentiation of ES cells. To explore this hypothesis, the functions of endogenous FGF-2 and/or BMP-2 were inhibited by the addition of anti-FGF-2 antibody, BMPR-1B/Fc chimera, and/or Noggin/Fc chimera for each of the 17 days, including the first 3 days (Fig 9A). In addition, anti-TGF- β or anti-PDGF antibody was used in the same way as an experimental control because it has been already shown that TGF- β and PDGF play important roles in the cardiomyogenic differentiation of ES cells.^{14,39}

Expression levels of Nkx2.5 on day 3+7 and day 3+14, MEF2C on day 3+14 and α MHC on day 3+7 were drastically inhibited by the addition of either of these inhibitors (Fig 9B). On the other hand, inhibition of the expression of GATA4 was relatively weak on day 3+7 or day 3+14 after the addition of any of inhibitors. Addition of non-specific antibodies, such as anti-mouse IgG, in the place of these inhibitors gave the same results as for the NC (data not shown). These results provided strong verification of our hypothesis; that endogenous FGF-2 and BMP-2 natively secreted from ES cells may play essential roles in the in vitro cardiomyogenic differentiation of ES cells. In addition, the expression of α MHC on day 3+14 was apparently but somewhat weakly inhibited by such inhibitors. Accordingly, the number of contracting clusters of ES cells was apparently decreased on day 3+7 and day 3+14, but small numbers of them were still observed on day 3+14. Thus, FGF-2 and BMP-2 may be more predominantly involved in up-regulation of Nkx2.5 and early cardiomyogenic differentiation than in later cardiomyogenic differentiation and maturation.

Discussion

This is the first study to successfully enhance the in vitro cardiomyogenic differentiation of ES cells using FGF-2 and BMP-2. The present results concerning FGF-2 are especially important and useful because there has not been a previous report of the role of FGF-2 in the cardiomyogenic differentiation of ES cells nor of its stronger efficacy than BMP-2. In addition, the effect of FGF-2 is likely to be additive to and independent of that of BMP-2. Thus, the present study provides important information for the elucidation of cardiac development and differentiation relating to FGF-2 and BMP-2, as well as for the development of transplantation therapy using ES cell-derived cardiomyocytes.

Based on the results from our preliminary studies, we decided to proceed with the present study using the approach of an initial extensive screening by semi-quantitative RT-PCR analyses and final verification with immunohistochemical and morphometric analyses. RT-PCR has the distinct advantage of facilitating analysis of numbers of samples; this was crucial for the present study that was investigating many factors and protocols, and experiments were repeated several times to verify reproducibility. On the other hand, PCR-based analysis has a semi-quantitative

nature, even though our experiments were carefully done with an internal control on the predetermined optimal PCR conditions and the results were confirmed by repeated experiments. In this regard, after the optimization by PCR-based screenings, the immunohistochemical and morphometric analyses finally verified the cardiomyogenic differentiation and accurately quantified the actual percentages of ES cell-derived cardiomyocytes. On the other hand, this analysis is time-consuming despite its advantage of accurate quantification and being the most reliable method. In addition, we also repeated these experiments several times to confirm reproducibility, and moreover, the double-blind test was done strictly to omit any subjective factors. Thus, this type of morphometric analysis is appropriate for the final verification, but not for extensive screening of multiple factors. On the other hand, some investigators have generated the ES cells that had stable expression of a marker gene under the control of each of the following promoters or transcriptional units of cardiac-specific genes (ie, cardiac α -actin, α MHC, Nkx2.5 or myosin light chain-2v)^{2,14,40-42} and the cardiomyogenic differentiation was quantified based on the marker-positive cells. We also generated ES clones that stably expressed a marker gene under the promoter of Nkx2.5 or α MHC, and compared the specificity and the reliability of both analyses (data not shown). As a result, the immunohistochemical and morphometric analyses of Nkx2.5, α MHC and tropomyosin-positive cells, as shown in the present report, were able to detect cardiomyocytes more reliably, correctly and specifically than the marker gene-based analyses, for the following reasons. First, tissue-specific promoters predominantly but not completely reproduce endogenous expression patterns; this lesser specificity may lead to false positive results. Second, the activities of tissue-specific promoters or transcriptional regulatory elements are often weak; the much lower sensitivity of this method than immunohistochemistry may also lead to false negative results. Thus, we carefully chose the strategy and approach for the present study.

One of the important findings was that optimal concentrations of FGF-2 and BMP-2 were indispensable for achieving efficient in vitro cardiomyogenic differentiation of ES cells; furthermore, such concentrations (1.0 ng/ml in FGF-2 and 0.2 ng/ml in BMP-2) were relatively lower than expected. Interestingly, dose-dependent effects of FGF-2 and BMP-2 for cardiac differentiation have been observed in chicken embryos.²¹ The maximal incidence of cardiogenic differentiation of non-precardiac mesoderm explanted from stage 6 avian embryos occurred at a concentration of 50 ng/ml of recombinant FGF-2 and BMP-2 proteins. The difference in the optimal concentrations of FGF-2 and BMP-2 between this previous study and our present one may be largely related to differences in species (mammalian vs non-mammalian cells) and experimental systems. There have been no previous studies in mammalian cells, including ES cells, that have clearly demonstrated the necessity of optimal concentrations of any growth factors, including FGF-2 and BMP-2, for maximal cardiomyogenic differentiation. Thus, the present paper is the first to reveal that cardiac differentiation may be tightly regulated by the optimal concentrations of FGF-2 and BMP-2 in mammals, or even in vitro.

We explored the expression levels of FGFRs and BMPRs to clarify, at least in part, this mechanism. The biological roles of individual FGFRs and BMPRs have not yet been

elucidated, and other types of cells derived from ES cells were contaminated; both facts may hamper further investigation of the detailed mechanisms within this study. However, down-regulations of all FGFRs and BMPRs, including FGFR-1, well-known predominant FGFR;³⁷ and BMPR-II, presumably predominant BMPR, were characteristically and remarkably seen in the case of the addition of FGF-2 or BMP-2 at higher concentrations. In this regard, one possible explanation of the optimal concentrations of FGF-2 and BMP-2 needed for the efficient cardiomyogenic differentiation may be down-regulation of their receptors in the case of addition of FGF-2 or BMP-2 at higher concentrations.

A recent study has shown the positive effect of TGF- β and BMP-2 for the cardiomyogenic differentiation of ES cells;⁴ but there has not been one for FGF-2. In that previous study, ES cells were pretreated with TGF- β (2.5 ng/ml) or BMP-2 (5.0 ng/ml) in 3.5% or 7.5% FCS-containing medium in the presence of LIF for 24 h, and then EBs were formed in the culture media containing 20% FCS without LIF. In contrast to those results, TGF- β (0.5–10 ng/ml) did not induce any apparent up-regulation of Nkx2.5 in the present study and moreover, our careful investigations clearly indicated that the optimal concentration of BMP-2 was 0.2 ng/ml, at least in our protocol. Such discrepancies between the previous study and the present one may be largely caused by the different protocols and different kinds of ES cells. Especially, they exposed ES cells to TGF- β only in the undifferentiated state (ie, only before the formation of EBs) as the pretreatment, and the concentrations of FCS before and during the differentiation were completely different from ours. In addition, it should be noted that the efficiency of the cardiomyogenic differentiation of the control ES cells in the previous study is likely to be much lower than ours (<10% in theirs and >20% in ours). Variance may partially result from different methods among studies; there may be both the possibility of underestimation because of false-negative cells in the previous studies, as described earlier, and somewhat overestimation because of overlapped cells in the present study. However, a more likely and possible explanation is that the endogenous levels of BMP-2 and TGF- β may have been originally higher in our system than in theirs. The fact of such diversities resulting from individual ES cell lines and protocols implies the necessity for future extensive investigations using several types of human ES cell lines for the purpose of development of cell therapy. There has been only one previous study which very roughly investigated the effects of growth factors on the differentiation of cells derived from human ES cells; only 10 ng/ml of FGF-2 was used, and cardiac differentiation was analyzed only by RT-PCR of cardiac actin.⁹ In this regard, it will be interesting and important to carefully investigate the optimal concentrations of FGF-2 and BMP-2 for inducing the maximal cardiomyogenic differentiation of human ES cells based on the present results.

Another important finding in the present study was that exposure to FGF-2 and BMP-2 for the appropriate period (ie, only during the first 3 days while EBs were being formed) led to efficient cardiomyogenic differentiation. This period may correspond to the time between embryonic day 4 (E4) and E6, when the 3 germ layers are being formed. A recent study of mice deficient for BMP-2 suggested that BMP-2 is a critical factor for both extraembryonic and embryonic development; notably, BMP-2-deficient embryos

exhibited a defect in cardiac development and died of cardiac and mesodermal defects between E7.0 and E10.5.²³ In addition, it should be noted in the present study that exposure to BMP-2 on the days after the first 3 actually diminished the cardiomyogenic differentiation of ES cells. Similarly, it has been reported that cardiac development in chicken embryos was efficiently induced by exposure to BMP-2 or FGF-2 for only 30 min.²¹ Taking these results together, we consider that FGF-2 and BMP-2 may play crucial roles in the induction and/or the early stage of in vitro cardiomyogenic differentiation of ES cells; thus, exposure of ES cells to FGF-2 and BMP-2 for no more than the appropriate period may efficiently enhance cardiomyogenic differentiation.

When administered alone, FGF-2 or BMP-2 failed to induce cardiac development in chicken embryos, suggesting that cooperative effects of FGF-2 and BMP-2 were necessary for the induction of cardiac differentiation.²¹ However, in the present study cardiac differentiation was apparently enhanced by the addition of FGF-2 alone or BMP-2 alone, although FGF-2 and BMP-2 in combination revealed the most prominent effectiveness. This inconsistency between studies may be related to differences in species and developmental systems. For example, mice lacking FGF-2 demonstrated normal embryonic development in spite of neuronal defects and delayed wound healing, suggesting a redundancy of FGF signaling in some tissues by other FGF family members in these mice.⁴³ The other possibility is that the inconsistency was related to the effect of endogenous FGF-2 and BMP-2, which may be secreted from certain types of differentiated ES cells, such as adjacent endoderm cells. We verified this hypothesis by experiments using inhibitors; inhibition of either FGF-2 or BMP-2 was in fact sufficient to drastically inhibit transcription of Nkx2.5.

In conclusion, FGF-2 and BMP-2 each play a crucial role in early cardiomyogenic differentiation. The present results, including the successful enhancement of the in vitro cardiomyogenic differentiation of ES cells using recombinant FGF-2 and/or BMP-2, may be highly useful for developing ES cell-based therapy for heart disease in humans, and for the basic study of cardiac development.

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