/0

Journal of the American College of Cardiology
© 2004 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 43, No. 10, 2004
ISSN 0735-1097/04/$30.00
doi:10.1016/j.jacc.2004.01.034

Powerful and Controllable Angiogenesis by Using Gene-Modified Cells Expressing Human Hepatocyte Growth Factor and Thymidine Kinase

Yasuyo Hisaka, MS,* Masaki Ieda, MD,† Toshikazu Nakamura, PHD,‡ Ken-ichiro Kosai, MD, PHD,§
Satoshi Ogawa, MD, PHD,† Keiichi Fukuda, MD, PHD*†

Tokyo, Osaka, and Fukuoka, Japan

OBJECTIVES This study investigated the possibility of achieving angiogenesis by using gene-modified cells as a vector.

BACKGROUND Although gene therapy for peripheral circulation disorders has been studied intensively, the plasmid or viral vectors have been associated with several disadvantages, including unreliable transfection and uncontrollable gene expression.

METHODS Human hepatocyte growth factor (hHGF) and thymidine kinase (TK) expression plasmids were serially transfected into NIH3T3 cells, and permanent transfectants were selected (NIH3T3 + hHGF + TK). Unilateral hindlimb ischemia was surgically induced in BALB/c nude mice, and cells were transplanted into the thigh muscles. All effects were assessed at four weeks.

RESULTS The messenger ribonucleic acid expression and protein production of hHGF were confirmed. Assay of growth inhibition by ganciclovir revealed that the 50% (median) inhibitory concentration of NIH3T3 + hHGF + TK was 1,000 times lower than that of NIH3T3 + hHGF. The NIH3T3 + hHGF + TK group had a higher laser Doppler blood perfusion index, higher microvessel density, wider microvessel diameter, and lower rate of hindlimb necrosis, as compared with the plasmid- and adenovirus-mediated hHGF transfection groups or the NIH3T3 group. The newly developed microvessels were accompanied by smooth muscle cells, as well as endothelial cells, indicating that they were on the arteriolar or venular level. Laser Doppler monitoring showed that the rate of blood perfusion could be controlled by oral administration of ganciclovir. The transplanted cells completely disappeared in response to ganciclovir administration for four weeks.

CONCLUSIONS Gene-modified cell transplantation therapy induced strong angiogenesis and collateral vessel formation that could be controlled externally with ganciclovir. (J Am Coll Cardiol 2004;43: 1915–22) © 2004 by the American College of Cardiology Foundation

Growth factors isolated recently, including vascular endothelial cell growth factor, fibroblast growth factor, angiopoietin, and hepatocyte growth factor (HGF), have been found to induce strong angiogenesis (1–5). A number of studies have reported induction of angiogenesis and collateral vessel formation by gene therapy with these factors in both animal experiments and clinical trials. Plasmid or viral vectors have been used in these therapies (2,6,7), but the adenovirus vector entails some serious problems, such as allergic reactions or difficulty with repeated treatment, despite sufficiently high transfection efficiency. Moreover, although plasmid vectors have recently been used in clinical settings, have not been associated with allergic reactions, and could be used repeatedly, their transfection efficiency has been low and has varied with the tissues injected or the patient. These gene delivery methods have the common drawbacks of not being able to choose the target cells and to selectively eliminate the transfected cells once they acquire the character of abnormal growth. Thus, new methods that would provide ideal gene delivery systems have long been awaited.

Regeneration therapy has recently been performed in many tissues and organs. Various types of cells regenerate from embryonic or adult stem cells, and these cells would be transplanted into patients. Rapid and sufficient establishment of angiogenesis and collateral vessel formation to promote the survival and function of the transplanted cells are especially important in terms of blood supply. We investigated regeneration of cardiomyocytes from adult stem cells and concluded that blood vessel formation into transplanted cells is crucial to their survival (8). Because angiogenic gene therapy with plasmid vectors has been insufficient to induce the rapid and powerful angiogenesis required for transplantation of the regenerated cells, a new method has been needed to address this problem.

In the present study, NIH3T3 cells were permanently transfected with a novel angiogenic human HGF (hHGF) and thymidine kinase (TK) of herpes simplex gene and then used as a gene therapy vector. Their effect on blood flow,

From the *Institute for Advanced Cardiac Therapeutics, †Cardiopulmonary Division, Department of Medicine, Keio University School of Medicine, Tokyo; ‡Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medicine, Osaka; and the §Cognitive and Molecular Research Institute of Brain Disease, Kurume University School of Medicine, Fukuoka, Japan. This study was supported in part by research grants from the Ministry of Education, Science and Culture, Japan, and by Health Science Research Grants for Advanced Medical Technology from the Ministry of Welfare, Japan.

Manuscript received June 28, 2003; revised manuscript received December 10, 2003, accepted January 5, 2004.

Abbreviations and Acronyms

DMEM	= Dulbecco's modified Eagle's medium
EGFP	= enhanced green fluorescent protein
ELISA	= enzyme-linked immunosorbent assay
hHGF	= human hepatocyte growth factor
IC_{50}	= 50% (median) inhibitory concentration
LDPI	= laser Doppler perfusion image
RT-PCR	= reverse transcription-polymerase chain reaction
SMA	= smooth muscle actin
TK	= thymidine kinase
vWF	= von Willebrand factor

angiogenesis, and collateral formation was investigated in a murine ischemic hindlimb model (9–11). In this paper, we report that gene-modified cells expressing hHGF and TK induced strong angiogenesis and collateral vessel formation, and that they were easily controlled externally with ganciclovir.

METHODS

Cell culture. The NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and penicillin (100 μg/ml), streptomycin (250 ng/ml), and amphotericin B (85 μg/ml).

Stable transfection of hHGF and TK genes. The complementary deoxyribonucleic acid (cDNA) of the hHGF and TK genes was inserted into the pUC-SRα and pGK expression vector plasmids, respectively (10–13). pPUR and pcDNA3.1/Hygro(+) are selection plasmids that confer puromycin resistance and hygromycin resistance, respectively. After co-transfection of pUC-SRα/hHGF and pPUR into the NIH3T3 cells, using the Effectene Reagent (QIAGEN GmbH, Hilden, Germany), the puromycin-nonresistant cells were removed with puromycin (3 μg/ml), and the hHGF-producing cells were clonally selected (NIH3T3 + hHGF). pGK/TK and pcDNA3.1/Hygro(+) plasmids were then similarly co-transfected into the NIH3T3 + hHGF cells; the hygromycin-nonresistant cells were removed with hygromycin (200 μg/ml); and both hHGF- and TK-producing cells were clonally selected (NIH3T3 + hHGF + TK).

Reverse transcription-polymerase chain reaction (RT-PCR). Expression of hHGF messenger ribonucleic acid was analyzed by RT-PCR using the primers that specifically detect human but not mouse HGF, as previously described (14).

Enzyme-linked immunosorbent assay (ELISA) for hHGF. Production of hHGF was determined by ELISA with anti-human–specific HGF monoclonal antibodies (Institute of Immunology, Tokyo, Japan) (6,15,16).

Ad.CA-hHGF. The adenoviral vector plasmid pAd.CA-hHGF, which is composed of a cytomegalovirus immediate early enhancer, a modified chicken beta-actin promoter, and hHGF cDNA, was constructed by the in vitro ligation method (17). The pAd.CA-hHGF plasmid was partially cut with *PacI* and then transfected into 293 cells, followed by culture with 0.5% overlaid agarose-α-minimal essential medium (MEM) containing 5% horse serum for 10 to 15 days. Viral plaques, which had been confirmed by restriction enzyme analysis and ELISA for hHGF, were propagated in 293 cells, purified by $CsCl_2$ gradient ultracentrifugation twice, and desalted with a desalting column (18). Viral particles were calculated by means of optical density at 260 nm.

Murine model of hindlimb ischemia. All animal experiments were approved by the Animal Care and Use Committee of Keio University. After anesthetizing male BALB/c nude mice (eight weeks) with diethyl ether, the femoral artery was gently isolated, and the proximal portion was ligated with 7-0 silk ligatures (19,20).

Transplantation of continuously hHGF-producing NIH3T3 cells. The hindlimb ischemic mice (n = 192) were randomly classified into five groups. The control groups received 0.2 ml saline only (n = 14), 500 μg pUC-SRα/hHGF plasmids in 0.2 ml saline (n = 10), 10^9 particles Ad.CA-hHGF in 0.2 ml phosphate-buffered saline (n = 10), or NIH3T3 in 0.2 ml DMEM (n = 14). The experimental group received NIH3T3 + hHGF + TK in 0.2 ml DMEM (n = 144). All injections were given via a 27-gauge needle (21). The numbers of cells transplanted ranged from 10^4 to 10^7. They were injected into two different sites in the ischemic thigh (adductor) skeletal muscles on postoperative day 1. The direction of injection was parallel to the muscle fibers. Angiogenesis and collateral vessel formation were assessed at four weeks.

Laser Doppler blood perfusion analysis. The blood perfusion rate in the ischemic (left leg) and normal (right leg) hindlimb was measured with a laser Doppler perfusion image (LDPI) system (Moor Instruments), as described previously (20,22).

Histopathology. Frozen sections (4 μm) were cut from tissue specimens (23). Immunohistochemical staining for hHGF, endothelial cells, and alpha-smooth muscle actin (SMA) was carried out with anti-human HGF (R&D Systems Inc., Minneapolis, Minnesota), anti-human von Willebrand factor (vWF)/horseradish peroxidase (HRP), and anti-human SMA/HRP (Dakocytomation, Kyoto, Japan), respectively. Sections for staining and counterstaining were incubated with 3,3′-diaminobenzidine tetrahydrochloride and Mayer's hematoxylin solution, respectively. Elastica van Gieson staining was carried out by the standard method. Paraffin sections (3 μm) were cut from tissue specimens, and hematoxylin-eosin staining was carried out by the standard method.

Assay of growth inhibition by ganciclovir in vitro. After seeding cells on six-well plates (10^5 cells/well) and culturing for 24 h, they were exposed to ganciclovir in concentrations ranging from 0 to 10^{-3} g/ml for 72 h (24,25).

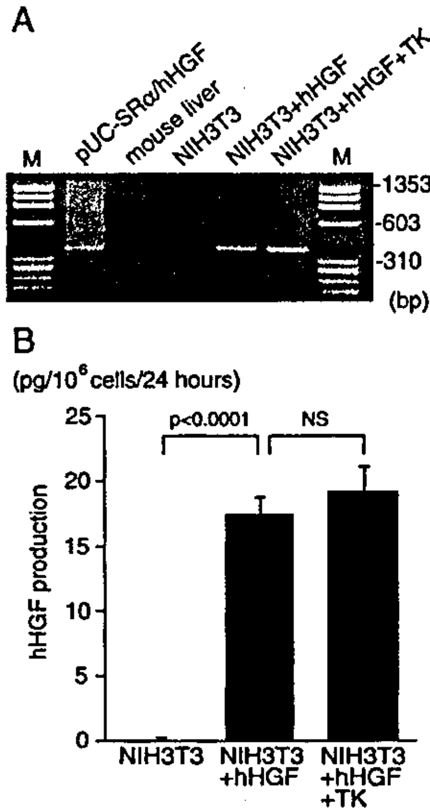

Figure 1. (A) Expression of human hepatocyte growth factor (hHGF) messenger ribonucleic acid in the hHGF-transfected NIH3T3 cells. The primer set of reverse transcription-polymerase chain reaction specifically detects hHGF but not mouse HGF. pUC-SRα/hHGF plasmid and mouse liver were used as a positive and negative control, respectively. M = the ΦX174-*Hae*III digest. (B) Production of hHGF protein. This ELISA system specifically detects only hHGF because of the lack of cross-reactivity by the antibodies. Data are expressed as hHGF concentrations adjusted for cell number. Both NIH3T3 + hHGF and NIH3T3 + hHGF + thymidine kinase (TK) groups expressed hHGF messenger ribonucleic acid and produced hHGF protein (n = 5).

Detection of ganciclovir-induced apoptosis with annexin V. Annexin V is an early apoptotic marker. The NIH3T3 + hHGF + TK group was exposed to 10^{-7} g/ml ganciclovir for 48 h, and the apoptotic cells were detected with an annexin V-enhanced green fluorescent protein (EGFP) apoptosis detection kit (Medical & Biological Labs Co. Ltd., Nagaya, Japan) (26).

Regulation of transplanted cell growth with ganciclovir in vivo. We investigated the dose-response relationship of growth inhibition by ganciclovir by transplanting NIH3T3 + hHGF + TK (10^7 cells) and administering ganciclovir two weeks later. The transplanted mice received different doses (0, 1, 10, 50, or 80 mg/kg per day) of ganciclovir orally once a day for four weeks.

Statistical analysis. The data were processed using StatView J-4.5 software. Results are reported as the mean value ± SE. Comparisons of values among all groups were performed by one-way analysis of variance. The Scheffe's F test was used to determine the level of significance. The probability level accepted for significance was $p < 0.05$.

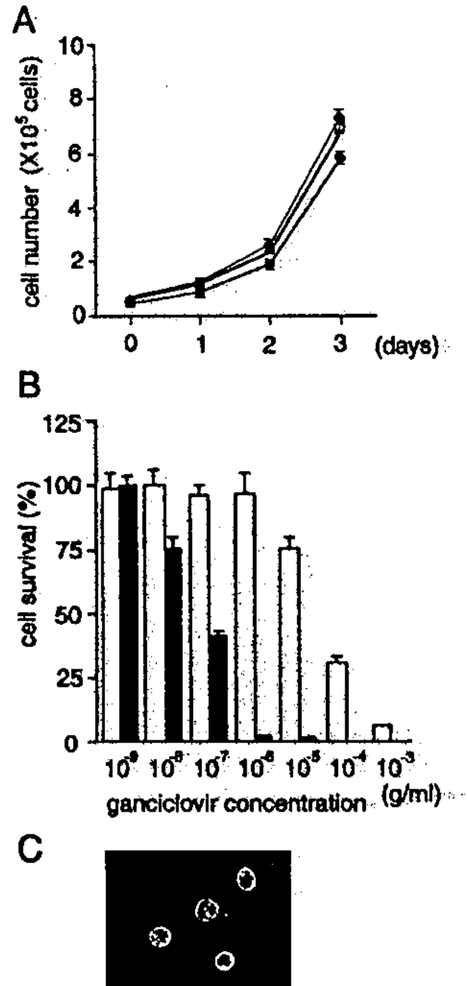

Figure 2. (A) Influence of hHGF and/or TK genes on cell growth in vitro. The growth rate of the hHGF-transfected NIH3T3 cells was slightly higher than that of the nontransfected cells, but TK had no effect on cell growth. (circles = NIH3T3; diamonds = NIH3T3 + hHGF; squares = NIH3T3 + hHGF + TK) (n = 3). (B) Growth-inhibitory effect of ganciclovir. The IC_{50} of ganciclovir for the NIH3T3 + hHGF + TK group (solid bars) was ~1,000 times lower than that for the NIH3T3 + hHGF group (open bars) (n = 5). (C) Apoptotic cells stained with annexin V-EGFP at the cell membrane after exposure to ganciclovir. Abbreviations as in Figure 1.

RESULTS

Permanently hHGF-transfected NIH3T3 cells produced hHGF protein. The NIH3T3 + hHGF cells were obtained after two weeks of exposure to puromycin, and NIH3T3 + hHGF + TK cells were obtained after two more weeks of exposure to hygromycin. We confirmed that both the NIH3T3 + hHGF and NIH3T3 + hHGF + TK groups expressed hHGF mRNA and then produced hHGF protein at a rate of 17.3 ± 1.4 and 19.1 ± 2.0 pg/10^6 cells per 24 h, respectively (Fig. 1).

Ganciclovir-inhibited cell growth and induced apoptotic cell death. It is well known that HGF regulates cell growth. To determine whether transfection of hHGF affects the growth of NIH3T3 cells, we counted the numbers

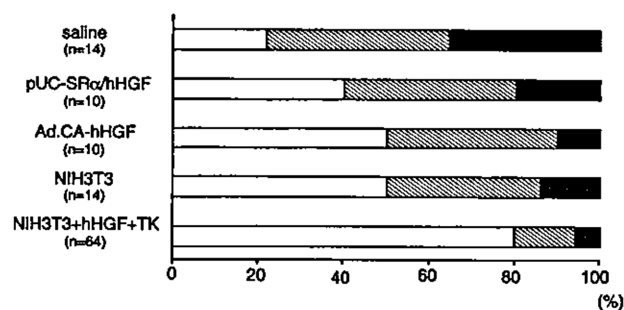

Figure 3. Frequency of necrosis in the ischemic hindlimbs. Severe hindlimb necrosis was significantly reduced in the NIH3T3 + hHGF + TK group. **Open areas** = negative necrosis; **lined areas** = necrosis on toes; **solid areas** = necrosis on foot. Abbreviations as in Figure 1.

of cells in vitro (Fig. 2A). The growth rate of the hHGF-transfected NIH3T3 cells seemed to increase slightly, but the increase was not significant on day 3. Transfection of the TK gene had no effect on their growth rate.

Next, we investigated the growth-inhibitory effect of ganciclovir on these cells (Fig. 2B). The IC_{50} of ganciclovir for the NIH3T3 + hHGF + TK group was ~1,000 times lower than that for the NIH3T3 + hHGF group. These findings confirmed that the TK plasmid genes had been effectively transfected, and that hardly any of the cells that expressed the TK gene survived exposure to ganciclovir at a concentration of 10^{-6} g/ml, which did not affect the control cells.

Enhanced green fluorescent protein fluorescence was detected at the membranes of NIH3T3 + hHGF + TK cells after ganciclovir exposure (Fig. 2C), indicating that cell death was attributable to apoptosis.

Human HGF-producing cell therapy augmented angiogenesis and collateral vessel formation. To evaluate whether transplantation of hHGF-producing cells improves the perfusion of ischemic hindlimbs, we first determined the rate of necrosis of the ischemic hindlimb. Necrosis was rated on a three-grade scale. The rate of necrosis of the foot and toes in the saline group was 35.7% and 42.9%, respectively. The rates in the pUC-SRα/hHGF group were 20% and 40%, respectively, and in the Ad.CA-hHGF group 10% and 40%, respectively. These therapeutic approaches were effective in comparison with the saline group, but they were not sufficient to fully prevent the necrosis. To further ameliorate limb necrosis, we examined angiogenic gene-modified cell transplantation therapy. The NIH3T3 (10^7 cells) group had rates of 14.3% and 35.7%, respectively, suggesting that the vector cell transplantation itself might improve perfusion of the ischemic limb to some extent. In contrast, the rates in the NIH3T3 + hHGF + TK (10^7 cells) group were 5.8% and 14.5%, respectively (Fig. 3). The rate of necrosis was surprisingly reduced in the NIH3T3 + hHGF + TK group, indicating that transplantation of hHGF-producing cells might be one of the most effective methods of improving limb ischemia.

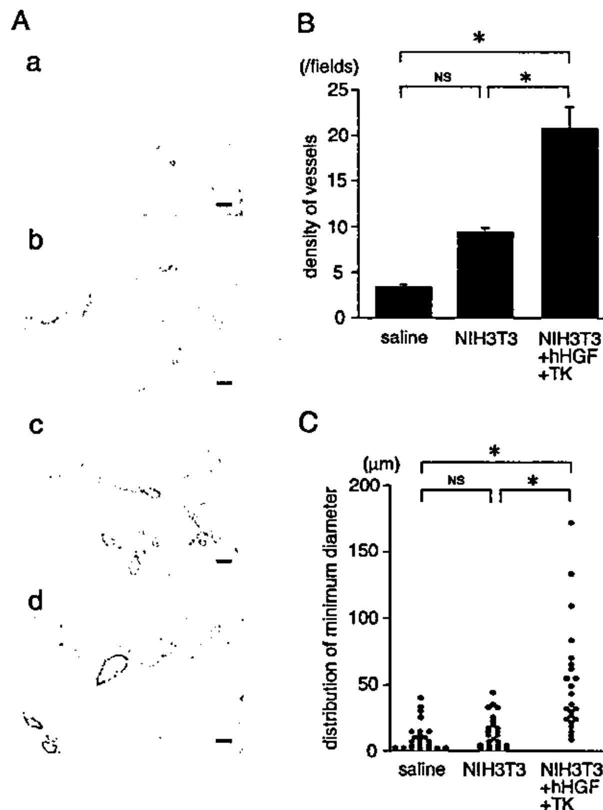

Figure 4. (A, panels a to d) Immunohistochemical staining for von Willebrand factor in the triceps muscle of the left calf revealed the presence of numerous vessels. Vessels were larger and more numerous in the NIH3T3 + hHGF + TK group **(panels c and d)** than in the saline **(panel a)** and NIH3T3 groups **(panel b)**. **Scale bars** = 100 μm. **(B)** The number of vessels was determined by observation of 20 random fields from 10 mice (2 fields per mouse; *p < 0.01). **(C)** Distribution of the minimum diameters of the von Willebrand factor-positive vessels (n = 25; *p < 0.0001). Abbreviations as in Figure 1.

Vessel density and size. Immunostaining clearly revealed the presence of numerous vessels in the NIH3T3 + hHGF + TK group (Fig. 4A, panel c) and a lower number of vessels in the saline (Fig. 4A, panel a) and NIH3T3 (Fig. 4A, panel b) groups. Quantitative analysis revealed that the vessel density in the ischemic region was significantly higher (Fig. 4B), and the minimum diameter of the vWF-positive vessels was significantly greater (Figs. 4A, panel d, and 4C) in the NIH3T3 + hHGF + TK group.

Vessel maturation. Maturation of the vessels was investigated by staining three consecutive frozen sections of ischemic skeletal muscle. Amazingly, most of the vessels in the NIH3T3 + hHGF + TK group were vWF/α-SMA–double positive (Figs. 5A, panels a and b, and 5B). However, there was no increase in elastic fiber-positive cells, as compared with the saline and NIH3T3 groups (Figs. 5A, panel c, and 5B). These findings showed that NIH3T3 + hHGF + TK cell transplantation strongly induced angiogenesis not only at the capillary level but also at the microvessel (arteriole) level, and it caused angiogenesis at the large blood vessel level.

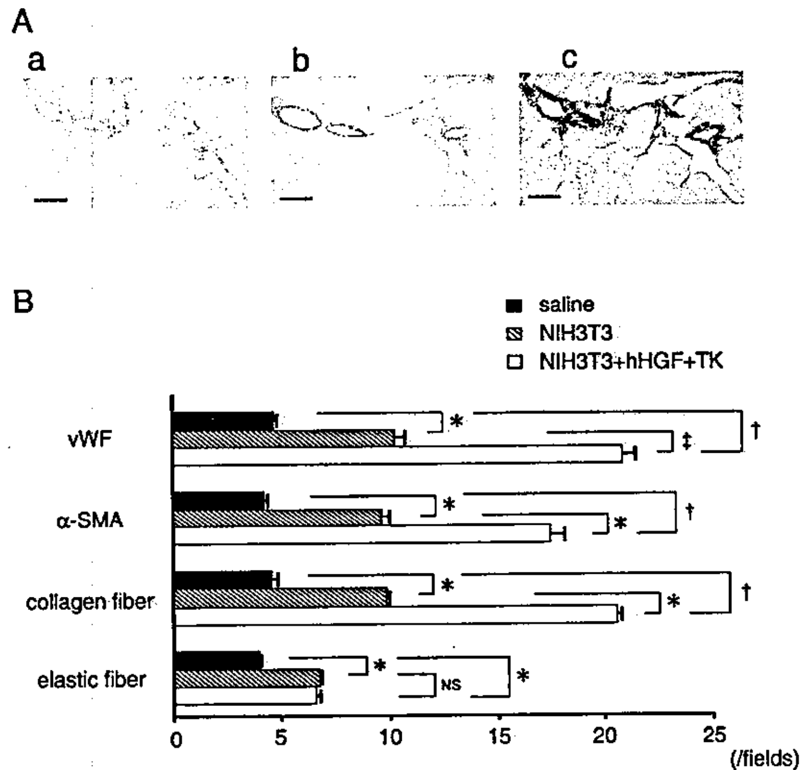

Figure 5. **(A, panels a to c)** Three consecutive frozen sections of NIH3T3 + hHGF + TK transplanted muscle. **(panel a)** Immunohistochemical staining for vWF and **(panel b)** α-smooth muscle actin (SMA) and **(panel c)** elastica van Gieson staining. **Scale bars** = 100 μm. **(B)** Maturation of vessels was compared by using three consecutive frozen sections. Most of the von Willebrand factor (vWF)-positive vessels in NIH3T3 + hHGF + TK transplanted mice also stained with α-SMA (n = 20; *$p < 0.05$, †$p < 0.001$, ‡$p < 0.01$). Abbreviations as in Figure 1.

Laser Doppler blood perfusion. The LDPI analysis was performed to study subcutaneous blood perfusion. Representative images are shown in Figure 6A, and quantitative analysis of blood perfusion is shown in Figure 6B. No blood perfusion was observed in the hindlimb immediately after femoral artery ligation (Fig. 6A, panel a). Perfusion of the proximal part of the thigh had recovered at four weeks in the saline and NIH3T3 groups, but perfusion distal to the heel joint had markedly decreased (Fig. 6A, panels b and c). In the NIH3T3 + hHGF + TK (10^4 cells) group, perfusion of the ischemic limb almost recovered to the control (nonischemic) level, but perfusion distal to the heel was slightly decreased compared with the control level (Fig. 6A, panel d). In the NIH3T3 + hHGF + TK (10^7 cells) group, perfusion of the ischemic limb was 118.1% (i.e., much greater than that in the control hindlimb) (Figs. 6A and 6B, panel e). To adjust the recovery of blood perfusion in the ischemic limb to the appropriate level, we transplanted NIH3T3 + hHGF + TK (10^7 cells), monitored the LDPI level, and began giving ganciclovir when blood perfusion reached the control level (two weeks). This method enabled us to adjust the blood perfusion rate in the ischemic limb to the same level as in the control limb (Figs. 6A and 6B, panel f).

When the NIH3T3 + hHGF + TK cells were transplanted into the normal nonischemic limb, the blood perfusion increased more than that in the control limb. Up to six weeks after transplantation, no evidence of angiosarcoma or hypervascular tumor was observed in the transplanted limb or other parts of the body (data not shown).

In vivo production of HGF protein. Immunohistochemical staining demonstrated the production of hHGF protein in transplanted NIH3T3 + hHGF + TK cells, but not in transplanted NIH3T3 cells (Fig. 7A).

Cell regulation with ganciclovir and TK. Figure 7B shows a quantitative analysis of the inhibitory effect of ganciclovir on blood perfusion. At a concentration of 50 mg/kg/day of ganciclovir, the blood perfusion was adjusted in the ischemic limb to the same level as in the control limb, and no significant side effects were produced. Histologic examination revealed the natural history of the transplanted cells (Fig. 7C, panels a to c). The transplanted cells formed a mass between the skeletal muscles, which gradually increased in size but did not infiltrate into the skeletal muscle. Two weeks after transplantation of the NIH3T3 + hHGF + TK cells, we began giving ganciclovir orally every day for two to four weeks and then examined tissue samples (Fig. 7C, panels d to f). The NIH3T3 + hHGF + TK cells gradually underwent apoptosis, and by four weeks, no transplanted cells could be detected. The surrounding muscle cells and the generated vessels were unaffected by ganciclovir.

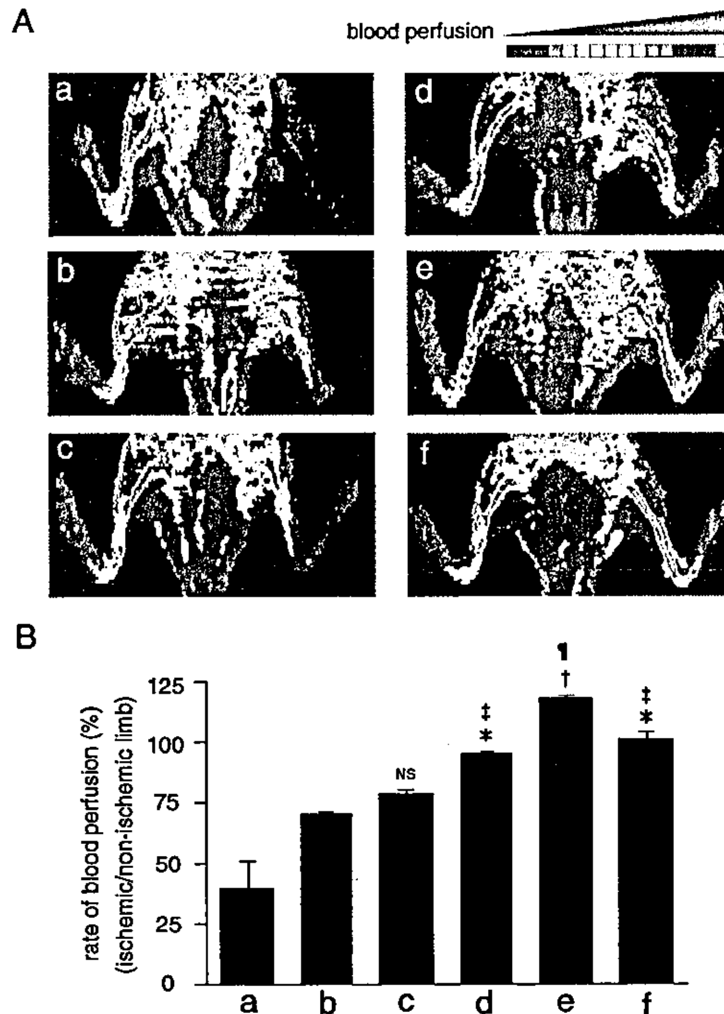

Figure 6. (A) Representative laser Doppler perfusion images. (B) Quantitative analysis of the rate of blood perfusion of the ischemic/nonischemic limb. **Panel a** = Control mouse on postoperative day 1; **panels b to f** = four weeks after treatment (**panel b** = saline injection; **panel c** = NIH3T3 transplantation [10^7 cells]; **panel d** = NIH3T3 + hHGF + TK transplantation [10^4 cells]; **panel e** = NIH3T3 + hHGF + TK transplantation [10^7 cells]; **panel f** = beginning two weeks after transplantation of NIH3T3 + hHGF + TK (10^7 cells), ganciclovir (50 mg/kg/day) was administered orally for four weeks. Oral ganciclovir administration adjusted the blood perfusion rate of the ischemic limb to the same level as that of the nonischemic limb (eight mice/group). *$p < 0.01$, †$p < 0.001$ vs. saline, ‡$p < 0.05$, ¶$p < 0.01$ versus NIH3T3. Abbreviations as in Figure 1.

DISCUSSION

In this study, we assessed angiogenic gene-modified cell transplantation therapy with fibroblasts permanently transfected with hHGF and TK genes in a murine hindlimb ischemia model. This therapy had the following merits: 1) it induced angiogenesis and collateral vessel formation more effectively than with plasmid and viral vectors. 2) The combination of TK and ganciclovir allowed the angiogenesis to be adjusted by monitoring LDPI. 3) This therapy could be stopped at any time desired for any reason. 4) There was no possibility of the hHGF gene being expressed in nontarget organs or nontarget cells as a result of leakage or dispersion of the vectors. If the plasmid vector was integrated into the genome and neoplastic transformation occurred, it would be difficult to control cell growth. 5) The angiogenic effect can be easily predicted, because the transfection efficiency of the gene is always 100%. 6) The cell vector will be much more effective in patients who require rapid angiogenesis, because plasmid or viral vectors require a week for maximal expression, and the duration of maximal expression is short.

Angiogenic gene-modified cell transplantation therapy has several drawbacks. One is that once the cells are transplanted into patients, their growth cannot be controlled. To solve this problem, we double-transfected the cells with the TK gene, and the results confirmed that permanently transfected cells could be killed with ganciclovir after the establishment of angiogenesis and collateral vessel formation. The finding that the IC_{50} of ganciclovir for the TK-transfected cells was 1,000 times lower than that in the nontransfected cells indicated that this system might be capable of being used in clinical settings.

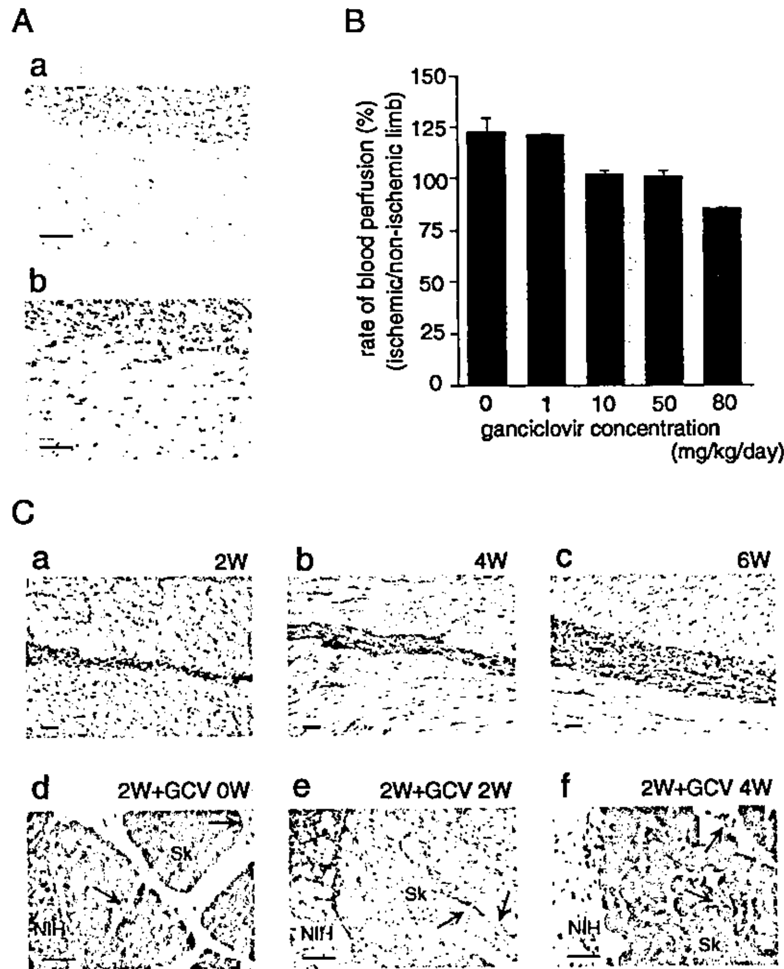

Figure 7. (A) Immunohistochemical staining for hHGF in transplanted NIH3T3 cells **(panel a)** and NIH3T3 + hHGF + TK cells **(panel b)** in the skeletal muscle. **Scale bars** = 50 μm. **(B)** The NIH3T3 + hHGF + TK (10^7) cells were transplanted, and two weeks later, various concentrations of ganciclovir were administered for another four weeks. **(C)** Hematoxylin-eosin staining. **(Panels a to c)** The natural history of the transplanted NIH3T3 + hHGF + TK (10^7) cells is shown. **(Panels d to f)** Beginning two weeks after transplantation, ganciclovir (50 mg/kg/day) was administered orally for two to four weeks. The cells had completely disappeared after four weeks of ganciclovir treatment. **Arrows** indicate the microvessels. **Scale bars** = 100 μm. Abbreviations as in Figure 1.

We used NIH3T3, a fibroblast line derived from fetal NIH/Swiss mice, for the following reasons: 1) the transfection efficiency of the plasmid is high; and 2) their growth rate is relatively high in vitro, making it easy to expand the cells. However, their growth rate in vivo is not as high as that of carcinoma cell lines, probably because NIH3T3 cells have a mechanism of growth inhibition by cell-cell contact. To apply this method in clinical medicine, the selection of a human cell line will be requried. Considering the time and cost for preparation of the cells, an autograft might require a long time and be expensive. It took at least two months to prepare the hHGF- and TK-double-transfected cells, and a number of additional experiments were needed to confirm their effectiveness and safety. We think that allograft cells should be used to prepare gene-modified cells. In view of the time, cost, effectiveness, and safety of the cells, allografts would be much better than autografts.

Regenerative medicine has recently been the subject of investigations in many fields, and a number of regenerative cells have been established. The authors have reported that regenerative cardiomyocytes can be generated from marrow mesenchymal stem cells, and transplantation of the regenerated cells will be examined in various organs. One of the reasons why we are considering angiogenic gene-modified cell transplantation therapy is the need for a rapid blood supply to the transplanted cells. To achieve that goal, we can co-transplant target organs with these gene-modified cells in combination with the regenerated cells. Once the blood supply has become established, the angiogenic cells are no longer needed, and they can be eliminated by ganciclovir.

Bone marrow mononuclear cells have recently been used to induce angiogenesis as a means of treating arteriosclerosis obliterans (27). Although bone marrow mononuclear cells contain endothelial cells, the population of endothelial progenitor cells is <1%. The effectiveness of this therapy may be explained not only by the presence of endothelial

progenitor cells but also by the fact that bone marrow mononuclear cells produce various cytokines and angiogenic growth factors. The advantage of angiogenic therapy with bone marrow mononuclear cell autografts is that the cells do not undergo immunorejection. The drawback of this therapy is that the cells may contain a variety of types of cells, such as osteogenic or chondrogenic stem cells, or induce inflammation by secreting cytokines. Using angiogenic gene-modified cells avoids the problem of transplanting different types of cells; however, the efficiency and safety of this procedure needs to be fully investigated before clinical application can become a reality.

Acknowledgments
The authors gratefully acknowledge Kensuke Kimura, MD, Isao Shibuya, PhD, and Haruko Kawaguchi, MS, for their kind assistance and helpful discussions.

Reprint requests and correspondence: Dr. Keiichi Fukuda, Institute for Advanced Cardiac Therapeutics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: kfukuda@sc.itc.keio.ac.jp.

REFERENCES

1. Isner JM, Pieczek A, Schainfeld R, et al. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 1996;348:370–4.
2. Yukawa H, Takahashi JC, Miyatake SI, et al. Adenoviral gene transfer of basic fibroblast growth factor promotes angiogenesis in rat brain. Gene Ther 2000;7:942–9.
3. Suri C, McClain J, Thurston G, et al. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998;282:468–71.
4. Morishita R, Nakamura S, Hayashi S, et al. Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. Hypertension 1999;33:1379–84.
5. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000;6:389–95.
6. Hayashi S, Morishita R, Nakamura S, et al. Potential role of hepatocyte growth factor, a novel angiogenic growth factor, in peripheral arterial disease: downregulation of HGF in response to hypoxia in vascular cells. Circulation 1999;100 Suppl II:II301–8.
7. Byun J, Heard JM, Huh JE, et al. Efficient expression of the vascular endothelial growth factor gene in vitro and in vivo, using an adeno-associated virus vector. J Mol Cell Cardiol 2001;33:295–305.
8. Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999;103:697–705.
9. Jainchill JL, Aaronson SA, Todaro GJ. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. J Virol 1969;4:549–53.
10. Nakamura T, Nishizawa T, Hagiya M, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature 1989;342:440–3.
11. McKnight SL. The nucleotide sequence and transcript map of the herpes simplex virus thymidine kinase gene. Nucleic Acids Res 1980;8:5949–64.
12. Hayashi S, Morishita R, Higaki J, et al. Autocrine-paracrine effects of overexpression of hepatocyte growth factor gene on growth of endothelial cells. Biochem Biophys Res Commun 1996;220:539–45.
13. Adra CN, Boer PH, McBurney MW. Cloning and expression of the mouse pgk-1 gene and the nucleotide sequence of its promoter. Gene 1987;60:65–74.
14. Ueda H, Sawa Y, Matsumoto K, et al. Gene transfection of hepatocyte growth factor attenuates reperfusion injury in the heart. Ann Thorac Surg 1999;67:1726–31.
15. Nakamura Y, Morishita R, Higaki J, et al. Expression of local hepatocyte growth factor system in vascular tissues. Biochem Biophys Res Commun 1995;215:483–8.
16. Yamada A, Matsumoto K, Iwanari H, et al. Rapid and sensitive enzyme-linked immunosorbent assay for measurement of HGF in rat and human tissues. Biomed Res 1995;16:105–14.
17. Mizuguchi H, Kay MA. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. Hum Gene Ther 1999;10:2013–7.
18. Chen SH, Chen XH, Wang Y, et al. Combination gene therapy for liver metastasis of colon carcinoma in vivo. Proc Natl Acad Sci USA 1995;92:2577–81.
19. Couffinhal T, Silver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM. Mouse model of angiogenesis. Am J Pathol 1998;152:1667–79.
20. Murohara T, Asahara T, Silver M, et al. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J Clin Invest 1998;101:2567–78.
21. Morishita R, Sakaki M, Yamamoto K, et al. Impairment of collateral formation in lipoprotein(a) transgenic mice: therapeutic angiogenesis induced by human hepatocyte growth factor gene. Circulation 2002;105:1491–6.
22. Shintani S, Murohara T, Ikeda H, et al. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation 2001;103:897–903.
23. Takeshita S, Zheng LP, Brogi E, et al. Therapeutic angiogenesis: a single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. J Clin Invest 1994;93:662–70.
24. Smee DF, Martin JC, Verheyden JP, Matthews TR. Anti-herpesvirus activity of the acyclic nucleoside 9-(1,3-dihydroxy-2-propoxymethyl) guanine. Antimicrob Agents Chemother 1983;23:676–82.
25. Cho HS, Park YN, Lyu CJ, et al. Effects of retroviral-mediated herpes simplex virus thymidine kinase gene transfer to murine neuroblastoma cell lines in vitro and in vivo. Acta Oncol 1999;38:1093–7.
26. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994;84:1415–20.
27. Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221–8.

Circ J 2004; **68:** 691–702

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

Takao Kawai, MD*; Tomoyuki Takahashi, PhD; Masayasu Esaki, MD*; Hiroaki Ushikoshi, MD*; Satoshi Nagano, MD†; Hisayoshi Fujiwara, MD*; Ken-ichiro Kosai, MD†,††

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.[3–6] 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)-β1 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.

(Received December 9, 2003; revised manuscript received April 23, 2004; accepted May 11, 2004)

*Department of Gene Therapy and Regenerative Medicine, Gifu University School of Medicine, *Cardiology, Respiratory and Nephrology, Regeneration & Advanced Medical Science, Graduate School of Medicine, Gifu University, Gifu, †Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University and ††Department of Pediatrics, Kurume University School of Medicine, Kurume, Japan*
Mailing address: Ken-ichiro Kosai, MD, PhD, Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan. E-mail: kosai@med.kurume-u.ac.jp

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 μmol/L 2-mercaptoethanol (2-ME), 1 μmol/L sodium pyruvate, 0.1 μmol/L nonessential amino acids and 10^3 unit/ml leuke-

Table 1 PCR Primers

	Sense	*Antisense*	*Annealing temperature (°C)*	*Reference*
Nkx2.5	*5'-CAGTGGAGCTGGACAAAGCC-3'*	*5'-TAGCGACGGTTCTGGAACCA-3'*	58	29
GATA4	*5'-CTGTCATCTCACTATGGGCA-3'*	*5'-CCAAGTCCGAGCAGGAATTT-3'*	58	29
MEF2C	*5'-AGCAAGAATACGATGCCATC-3'*	*5'-GAAGGGGTGGTGGTACGGTC-3'*	58	29
αMHC	*5'-GGAAGAGTGAGCGGCCATCAAGG-3'*	*5'-CTGCTGGAGGTTATTCCTCG-3'*	58	29
FGFR-1	*5'-GCTGACTCTGGCCTCTACGCT-3'*	*5'-CAGGATCTGGACATACGGCAA-3'*	62	17
FGFR-2	*5'-CTCCTTCAGTTTAGTTGAGGATACCA-3'*	*5'-GAAGATCCAAGTTTCACTGTC TACCG-3'*	60	17
FGFR-3	*5'-GAAGAATGGCAAAGAATTCCGAG-3'*	*5'-CCTCTAGCTCCTTGTCGGTGG-3'*	60	17
FGFR-4	*5'-GAACTCTCTGGGTAGCATTCGCT-3'*	*5'-TGTCTGTTGTCTTGAGGAC TTGTACG-3'*	58	17
BMPR-IA	*5'-TCGTCGTTGTATTACAGGAG-3'*	*5'-TTACATCCTGGGATTCAACC-3'*	58	30
BMPR-IB	*5'-GCTTTGGACTCATCCTCTGG-3'*	*5'-CACTGGGCAGTAGGCTAACG-3'*	54	30
ActR-I	*5'-AGATGACGTGTAAGACCCCG-3'*	*5'-ATACTTCTCCATAGCGGCCC-3'*	56	30
BMPR-II	*5'-GGTAGATAGGAGGGAACGGC-3'*	*5'-CACTGCCATTGTTGTTGACC-3'*	56	30
HPRT	*5'-CCTGCTGGATTACATTAAAGCACTG-3'*	*5'-AAGGGCATATCCAACAACAA-3'*	58	

MEF2C, myocyte enhancer factor 2C; αMHC, amyosin heavy chain; FGFR, fibroblast growth factor receptor; BMPR, bone morphogenetic protein receptor; ActR-I, activin receptor-I; HPRT, hypoxantine-phosphoribosyl-transferase.

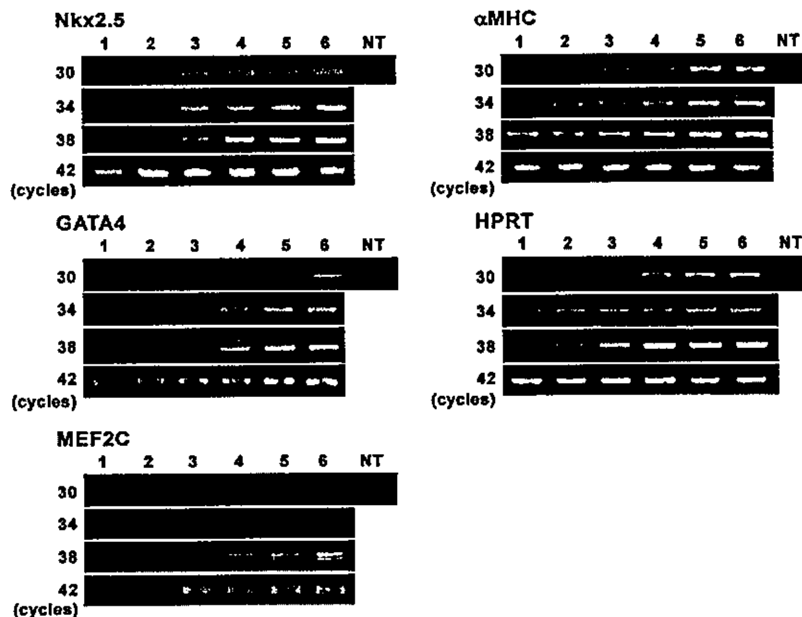

Fig 1. Optimization of the semi-quantitative RT-PCR assay. Serially diluted plasmid DNA containing Nkx2.5 cDNA (0.08, 0.15, 0.31, 0.63, 1.25 and 2.5 pg DNA in lanes 1, 2, 3, 4, 5 and 6, respectively) was used for control samples for semi-quantitative RT-PCR of Nkx2.5. Total RNA extracted from adult mouse 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 μmol/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 cm^2, 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-IB/Fc chimera,[27] 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 μg 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 30 s, each annealing temperature for 90 s and 72°C for 60 s 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

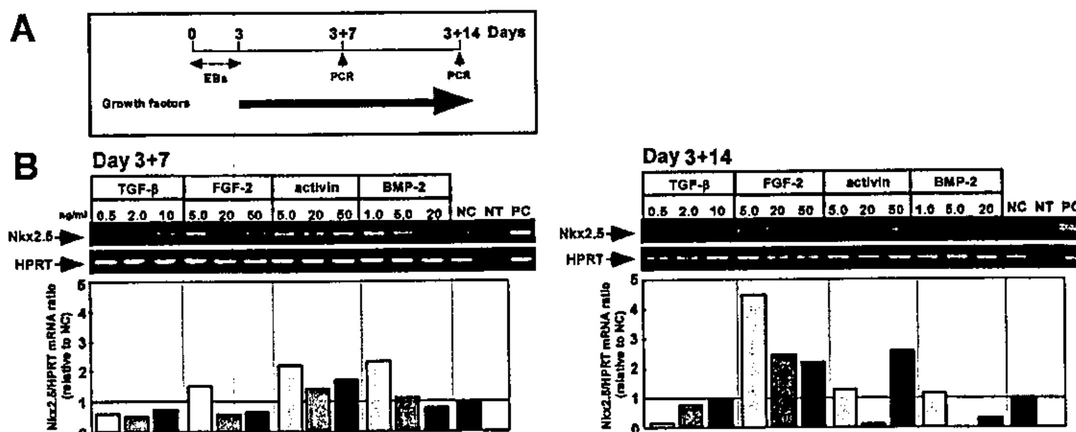

Fig 2. Nkx2.5 mRNA expression in ES cells treated with one of the 4 growth factors. (A) Experimental schedule. ES cells were cultured in suspension for 3 days to form EBs, and subsequently plated and cultured with media containing one of 4 recombinant growth factors at various concentrations for an additional 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 cardiomyocytes-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 ×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 ± 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

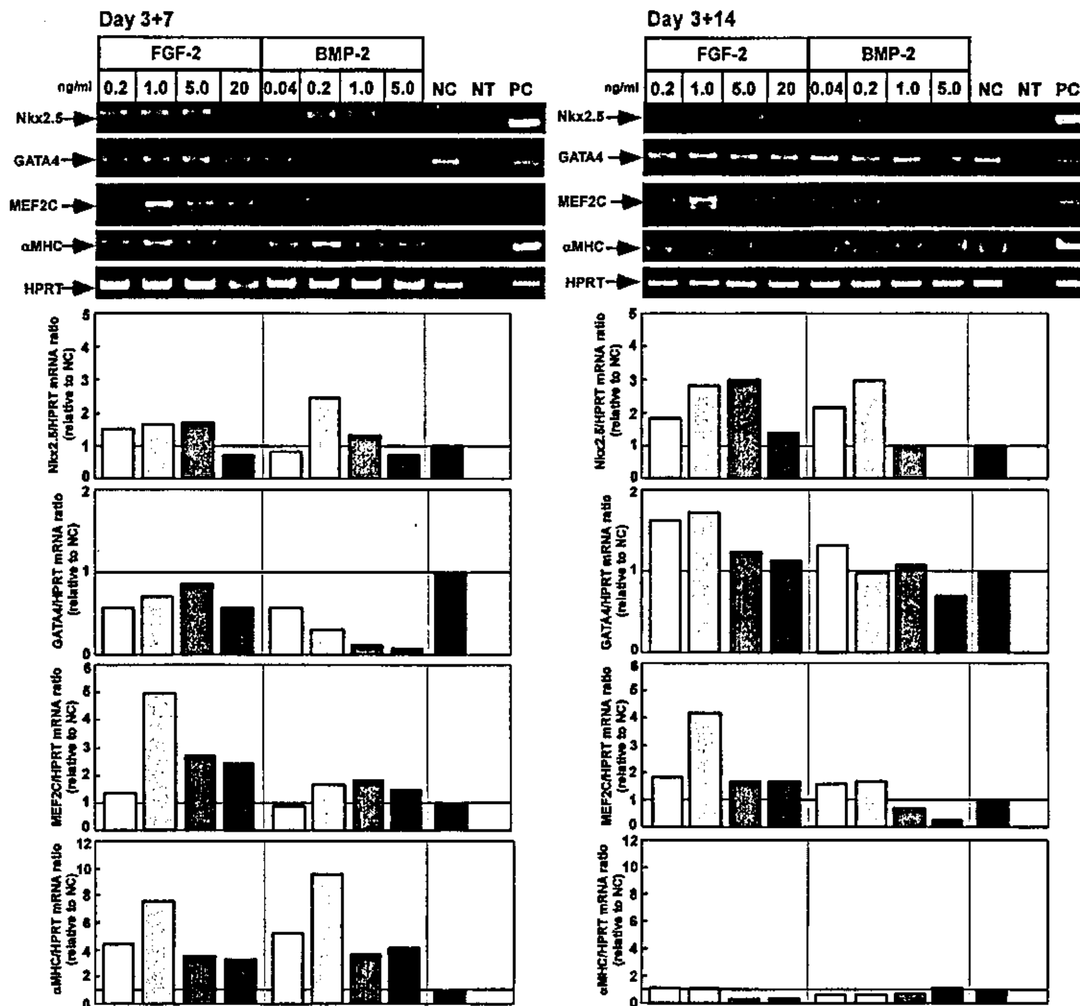

Fig 3. Effects of various doses of FGF-2 or BMP-2 on in vitro cardiomyogenic differentiation of ES cells. ES cells were cultured with FGF-2 or BMP-2 at various concentrations on the same schedule as shown in Fig 2A. RT-PCR analysis of cardiomyocyte-specific genes, Nkx2.5, GATA4, MEF2C and αMHC, was performed. The same results were obtained in 3 independent experiments. The Nkx2.5/HPRT, GATA4/HPRT, MEF2C/HPRT or αMHC/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 αMHC 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] αMHC is one of the representative sarcomeric proteins and thus is expressed in mature and differentiated cardiomyocytes.[36] In fact, expression of αMHC 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 αMHC 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

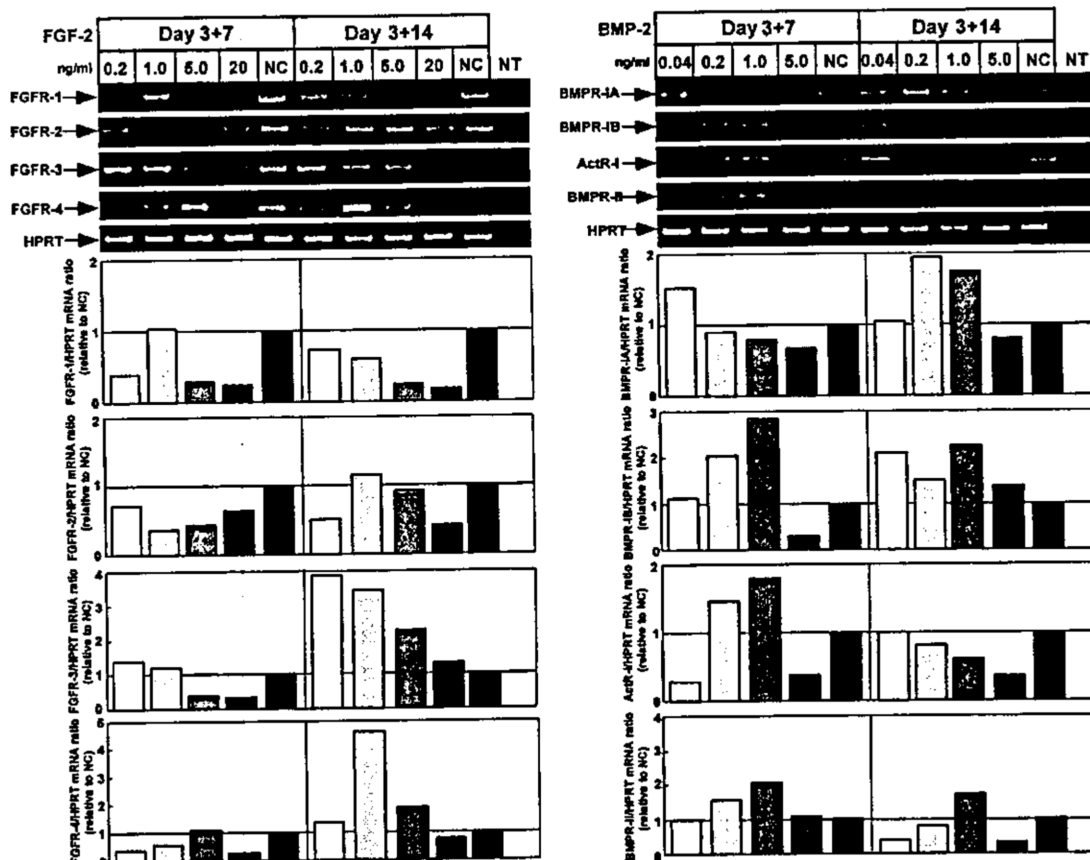

Fig4. Effects of various doses of FGF-2 or BMP-2 on the expression levels of FGFRs and BMPRs in ES cells. Experimental schedule was the same as shown in Fig2A. RT-PCR analyses of FGFRs (FGFR-1, FGFR-2. FGFR-3 and FGFR-4) and BMPRs (BMPR-IA, BMPR-IB, ActR-I and BMPR-II), and the calculation of FGFR/HPRT or BMPR/HPRT mRNA ratio were performed in the same way as shown in Fig3. The same results were obtained in 3 independent experiments.

a final concentration of 1.0–5.0ng/ml. The expression of αMHC 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 (0.2–20ng/ml); the most striking increase was seen in the case of 1.0ng/ml FGF-2. The expression of GATA4 was slightly increased on day 3+14 by additions of FGF-2 at a lower concentration (0.2–1.0ng/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 αMHC on day 3+7 was seen only when BMP-2 was added at a final concentration of 0.2ng/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 0.2ng/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 (0.04–1.0ng/ml). Thus, the optimal concentrations of FGF-2 and BMP-2 are 1.0 and 0.2ng/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 αMHC 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 Fig3, represent the ratio relative to the NC in each experiment, and that the actual expression level of αMHC in the NC was higher on day 3+14 than on day 3+7 (Fig3). 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 maturated 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

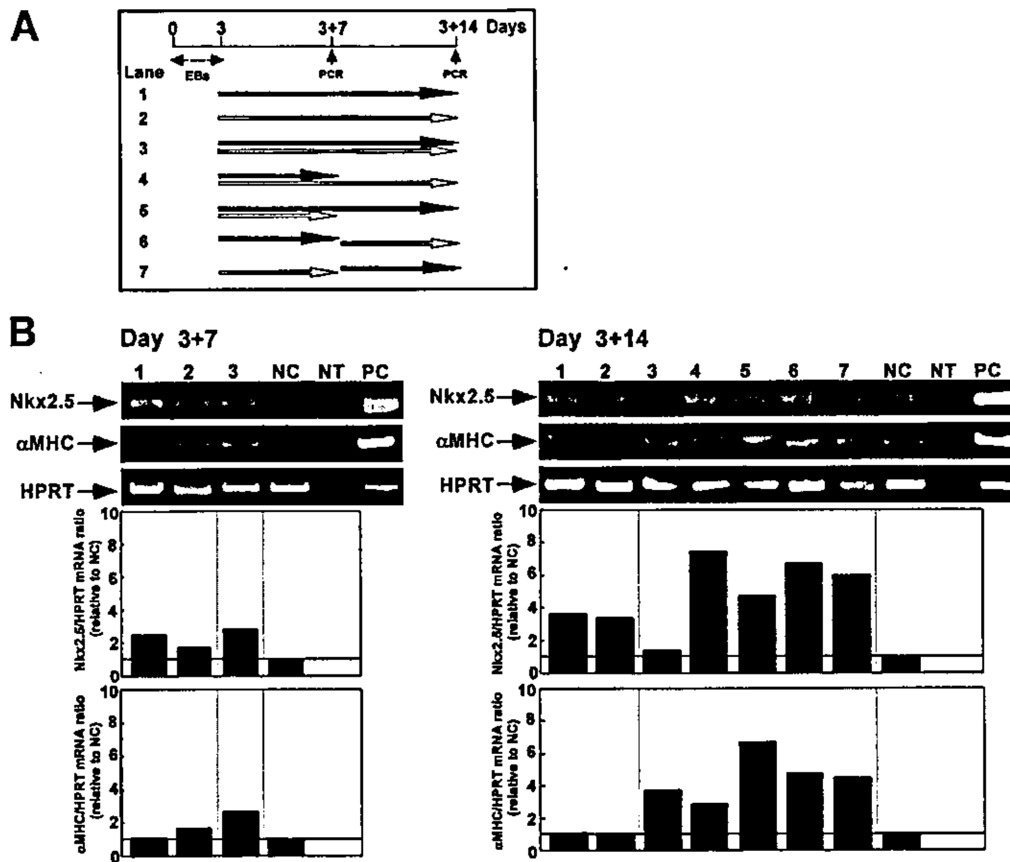

Fig 5. Effects of the combination of FGF-2 and BMP-2 on in vitro cardiomyogenic differentiation of ES cells. (A) Experimental schedules. ES cells were cultured with FGF-2 (1.0ng/ml) and BMP-2 (0.2ng/ml) following different schedules. Black and white arrows represent the addition of FGF-2 and BMP-2, respectively. (B) RT-PCR analysis of Nkx2.5 and αMHC was done and the Nkx2.5/HPRT or αMHC/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 cardiomyocytes.[38] 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 (1.0ng/ml in FGF-2 and 0.2ng/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 αMHC 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 αMHC 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 αMHC 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

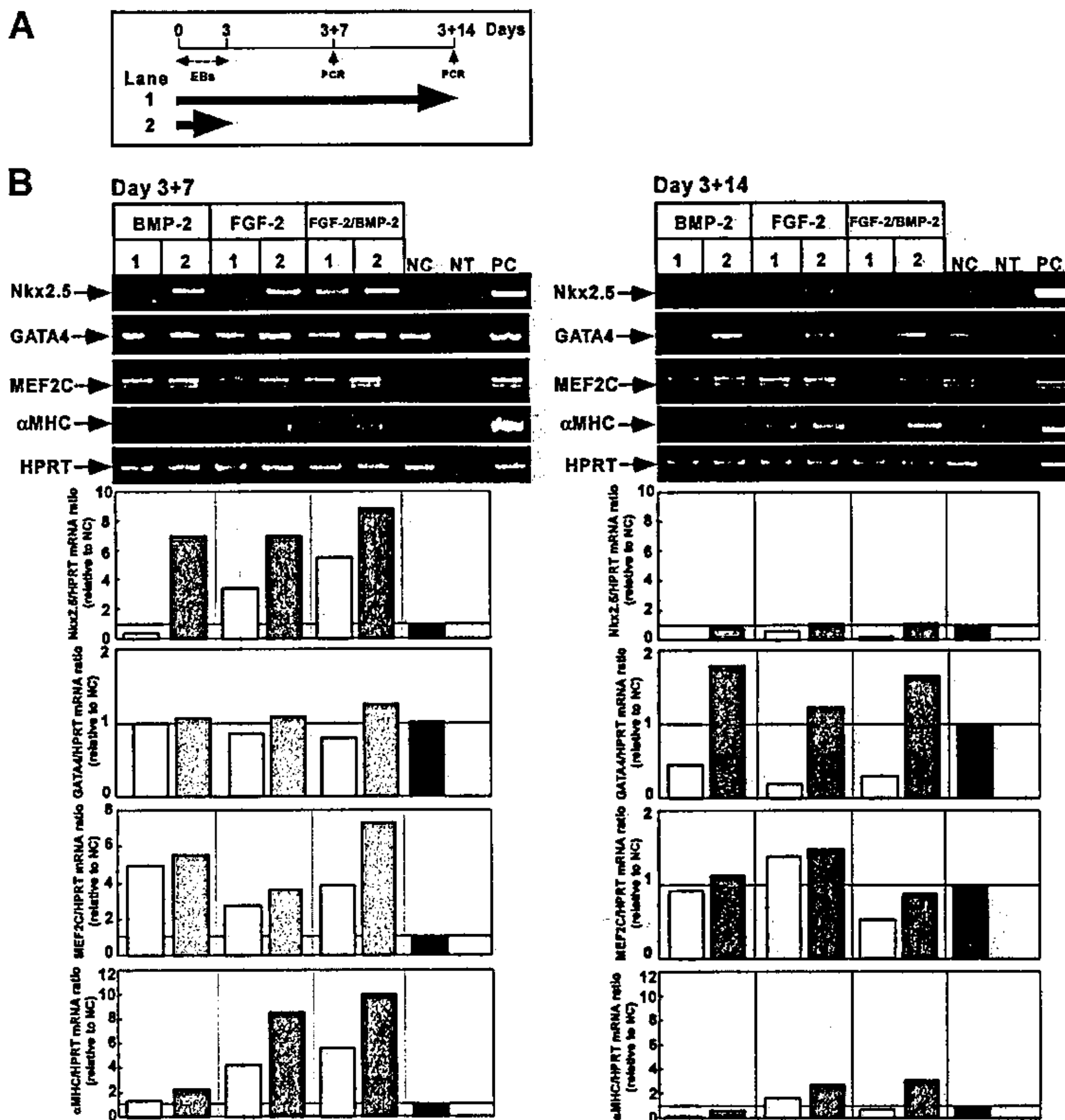

Fig 6. Efficacy of FGF-2 and BMP-2 for early cardiomyogenic differentiation. (A) Experimental schedules. In vitro differentiation was initiated by the formation of EBs without LIF in the same way as already shown, and FGF-2 (1.0 ng/ml) and/or BMP-2 (0.2 ng/ml) were added to the culture media either during 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 αMHC and the calculation of the Nkx2.5/HPRT, GATA4/HPRT, MEF2C/HPRT or αMHC/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 αMHC 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 αMHC 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 αMHC 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 αMHC 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.

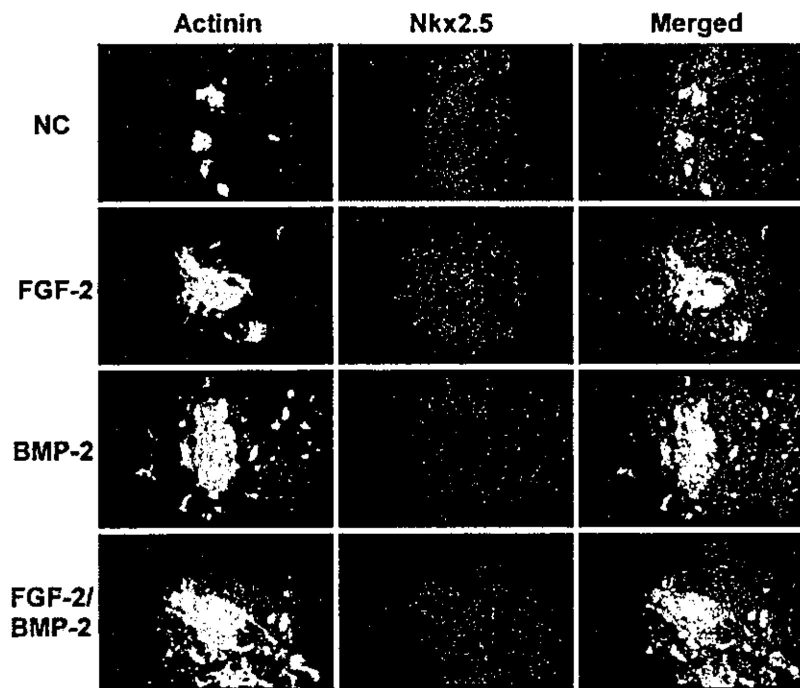

Fig7. Immunocytochemical staining of cardiomyocyte-specific genes, actinin and Nkx2.5. FGF-2 (1.0ng/ml) and/or BMP-2 (0.2ng/ml) were added for 7 days after the formation of EBs using the same protocol as shown in Fig5A. ES cells on day 3+7 were immunocytochemically stained with anti-actinin and anti-Nkx2.5 antibodies. NC, non-treatment control (Original magnification, ×100).

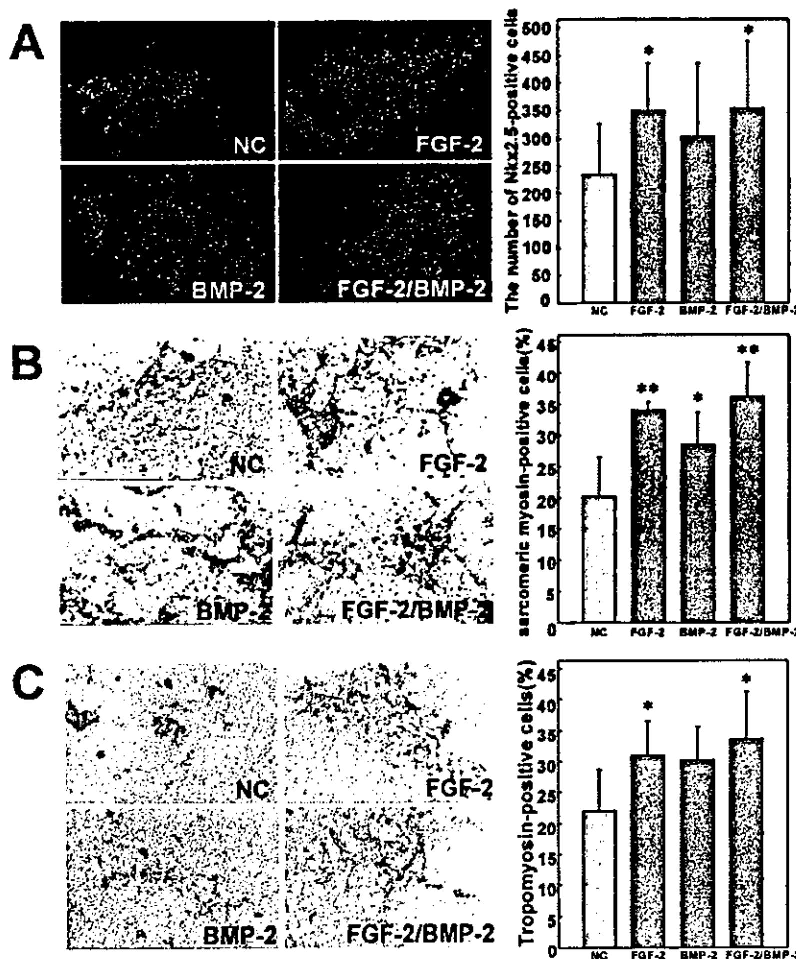

Fig8. Immunohistochemical and quantitative analysis of ES cell-derived cardiomyocytes. ES cells were cultured with FGF-2 (1.0ng/ml) and/or BMP-2 (0.2ng/ml) under the same protocol as shown in Fig5A. ES cells on day 3+7 were immunocytochemically stained with an antibody of anti-Nkx2.5 (A), anti-sarcomeric 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, ×100). *p<0.05; **p<0.001.

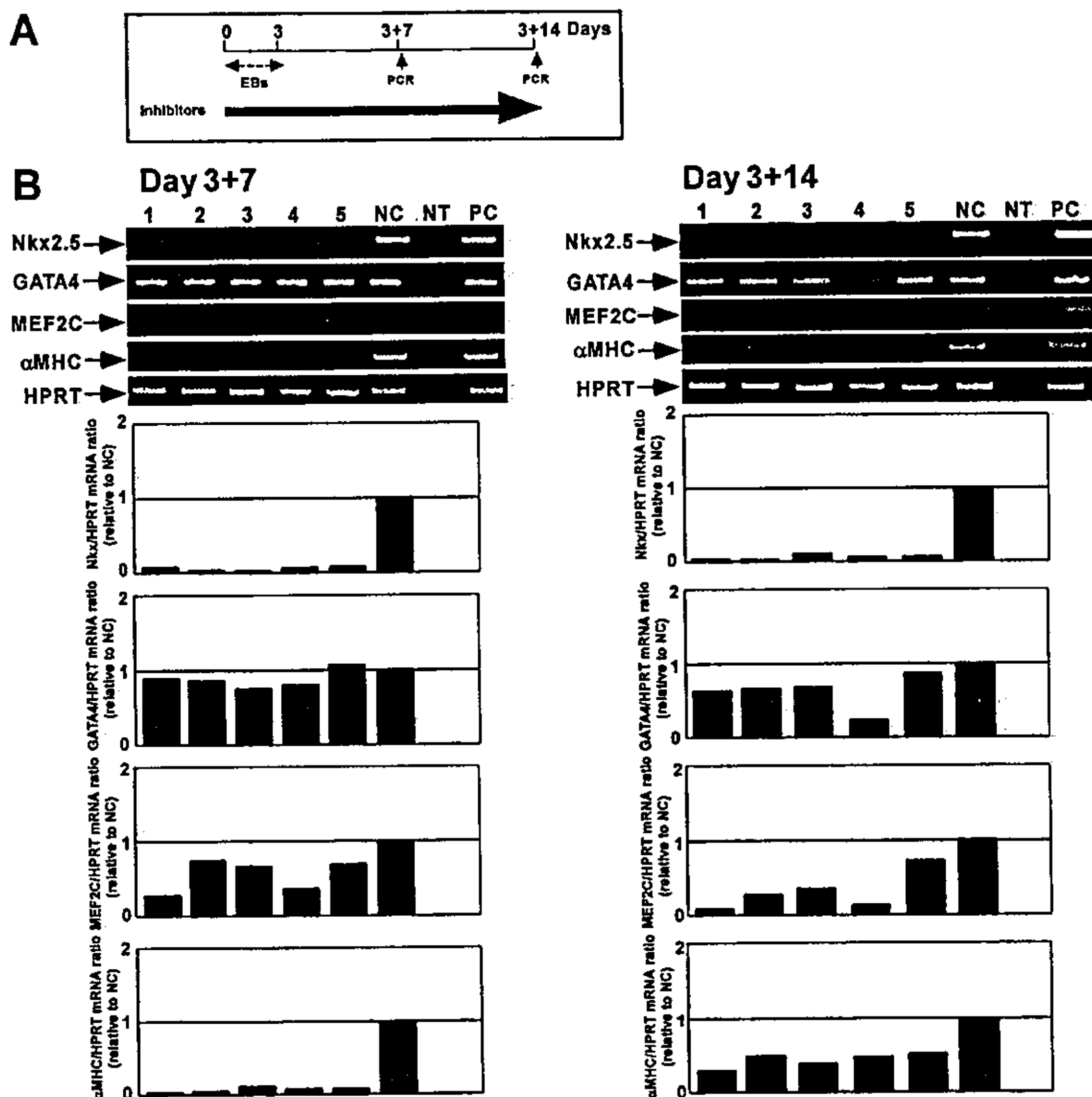

Fig 9. Inhibition of FGF-2 and BMP-2 attenuated in vitro cardiomyogenic differentiation of ES cells. (A) Experimental schedule. In vitro differentiation was initiated by the formation of EBs without LIF in the same way as already shown. Either anti-FGF-2 antibody (2.5μg/ml; lane 1), BMPR-1B/Fc (1.0μg/ml; lane 2), Noggin/Fc chimera (0.1μg/ml; lane 3), anti-TGF-β antibody (1.5μg/ml; lane 4) or anti-PDGF antibody (1.5μg/ml; lane 5) were added during the whole 3+7 or 3+14 days. (B) RT-PCR analysis of Nkx2.5, GATA4, MEF2C and αMHC, and the calculation of the Nkx2.5/HPRT, GATA4/HPRT, MEF2C/HPRT or αMHC/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 αMHC 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 αMHC 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, αMHC, 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 αMHC, and compared the specificity and the reliability of both analyses (data not shown). As a result, the immunohistochemical and morphometric analyses of Nkx.2.5, αMHC 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,[14] 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 cadiomyogenic 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.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science and a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology. We thank Dr A. Nagy for providing the R1 ES cells.

References

1. Johkura K, Cui L, Suzuki A, Teng R, Kamiyoshi A, Okamura S, et al. Survival and function of mouse embryonic stem cell-derived cardiomyocytes in ectopic transplants. *Cardiovasc Res* 2003; **58:** 435–443.
2. Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. *J Clin Invest* 1996; **98:** 216–224.
3. Sata M. Molecular strategies to treat vascular diseases: Circulating vascular progenitor cell as a potential target for prophylactic treatment of atherosclerosis. *Circ J* 2003; **67:** 983–991.
4. Koh GY, Soonpaa MH, Klug MG, Pride HP, Cooper BJ, Zipes DP, et al. Stable fetal cardiomyocyte grafts in the hearts of dystrophic mice and dogs. *J Clin Invest* 1995; **96:** 2034–2042.
5. Koh GY, Klug MG, Soonpaa MH, Field LJ. Differentiation and long-term survival of C2C12 myoblast grafts in heart. *J Clin Invest* 1993; **92:** 1548–1554.
6. Leor J, Patterson M, Quinones MJ, Kedes LH, Kloner RA. Transplantation of fetal myocardial tissue into the infarcted myocardium

of rat: A potential method for repair of infarcted myocardium? *Circulation* 1996; **94:** II-332–II-336.
7. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 2001; **108:** 407–414.
8. Heng BC, Haider H, Sim EK, Cao T, Ng SC. Strategies for directing the differentiation of stem cells into the cardiomyogenic lineage in vitro. *Cardiovasc Res* 2004; **62:** 34–42.
9. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. From the cover: Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 2000; **97:** 11307–11312.
10. Wiles MV, Keller G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* 1991; **111:** 259–267.
11. Biesecker LG, Emerson SG. Interleukin-6 is a component of human umbilical cord serum and stimulates hematopoiesis in embryonic stem cells in vitro. *Exp Hematol* 1993; **21:** 774–778.
12. Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. Embryonic stem cells express neuronal properties in vitro. *Dev Biol* 1995; **168:** 342–357.
13. Slager HG, Van Inzen W, Freund E, Van den Eijnden-Van Raaij AJ, Mummery CL. Transforming growth factor-beta in the early mouse embryo: Implications for the regulation of muscle formation and implantation. *Dev Genet* 1993; **14:** 212–224.
14. Behfar A, Zingman LV, Hodgson DM, Rauzier JM, Kane GC, Terzic A, et al. Stem cell differentiation requires a paracrine pathway in the heart *FASEB J* 2002; **16:** 1558–1566.
15. Olson EN, Srivastava D. Molecular pathways controlling heart development. *Science* 1996; **272:** 671–676.
16. Moore CS, Mjaatvedt CH, Gearhart JD. Expression and function of activin beta A during mouse cardiac cushion tissue formation. *Dev Dyn* 1998; **212:** 548–562.
17. Dell'Era P, Ronca R, Coco L, Nicoli S, Metra M, Presta M. Fibroblast growth factor receptor-1 is essential for in vitro cardiomyocyte development. *Circ Res* 2003; **93:** 414–420.
18. Choy M, Oltjen SL, Otani YS, Armstrong MT, Armstrong PB. Fibroblast growth factor-2 stimulates embryonic cardiac mesenchymal cell proliferation. *Dev Dyn* 1996; **206:** 193–200.
19. Schultheiss TM, Burch JB, Lassar AB. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev* 1997; **11:** 451–462.
20. Alsan BH, Schultheiss TM. Regulation of avian cardiogenesis by Fgf8 signaling. *Development* 2002; **129:** 1935–1943.
21. Barron M, Gao M, Lough J. Requirement for BMP and FGF signaling during cardiogenic induction in non-precardiac mesoderm is specific, transient, and cooperative. *Dev Dyn* 2000; **218:** 383–393.
22. Lough J, Barron M, Brogley M, Sugi Y, Bolender DL, Zhu X. Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precardiac embryonic mesoderm. *Dev Biol* 1996; **178:** 198–202.
23. Zhang H, Bradley A. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* 1996; **122:** 2977–2986.
24. Wood SA, Allen ND, Rossant J, Auerbach A, Nagy A. Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* 1993; **365:** 87–89.
25. Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res* 2002; **91:** 189–201.
26. Pirity M, Hadjantonakis AK, Nagy A. Embryonic stem cells, creating transgenic animals. *Methods Cell Biol* 1998; **57:** 279–293.
27. Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, Nakanishi S, et al. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* 2000; **28:** 31–40.
28. Lim DA, Tramontin AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* 2000; **28:** 713–726.
29. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* 1999; **103:** 697–705.
30. van der Horst G, van Bezooijen RL, Deckers MM, Hoogendam J, Visser A, Lowik CW, et al. Differentiation of murine preosteoblastic KS483 cells depends on autocrine bone morphogenetic protein signaling during all phases of osteoblast formation. *Bone* 2002; **31:** 661–669.
31. Gonzalez-Sanchez A, Bader D. Immunochemical analysis of myosin heavy chains in the developing chicken heart. *Dev Biol* 1984; **103:** 151–158.
32. Ikeda Y, Hiroi Y, Hosoda T, Utsunomiya T, Matsuo S, Ito T, et al. Novel point mutation in the cardiac transcription factor CSX/NKX2.5 associated with congenital heart disease. *Circ J* 2002; **66:** 561–563.
33. Tanaka M, Chen Z, Bartunkova S, Yamasaki N, Izumo S. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* 1999; **126:** 1269–1280.
34. Molkentin JD, Lin Q, Duncan SA, Olson EN. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev* 1997; **11:** 1061–1072.
35. Lin Q, Schwarz J, Bucana C, Olson EN. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* 1997; **276:** 1404–1407.
36. Morkin E. Control of cardiac myosin heavy chain gene expression. *Microsc Res Tech* 2000; **50:** 522–531.
37. Detillieux KA, Sheikh F, Kardami E, Cattini PA. Biological activities of fibroblast growth factor-2 in the adult myocardium. *Cardiovasc Res* 2003; **57:** 8–19.
38. Izumi M, Fujio Y, Kunisada K, Negoro S, Tone E, Funamoto M, et al. Bone morphogenetic protein-2 inhibits serum deprivation-induced apoptosis of neonatal cardiac myocytes through activation of the Smad1 pathway. *J Biol Chem* 2001; **276:** 31133–31141.
39. Sachinidis A, Gissel C, Nierhoff D, Hippler-Altenburg R, Sauer H, Wartenberg M, et al. Identification of plateled-derived growth factor-BB as cardiogenesis-inducing factor in mouse embryonic stem cells under serum-free conditions. *Cell Physiol Biochem* 2003; **13:** 423–429.
40. Hidaka K, Lee JK, Kim HS, Ihm CH, Iio A, Ogawa M, et al. Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. *FASEB J* 2003; **17:** 740–742.
41. Muller M, Fleischmann BK, Selbert S, Ji GJ, Endl E, Middeler G, et al. Selection of ventricular-like cardiomyocytes from ES cells in vitro. *FASEB J* 2000; **14:** 2540–2548.
42. Kolossov E, Fleischmann BK, Liu Q, Bloch W, Viatchenko-Karpinski S, Manzke O, et al. Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. *J Cell Biol* 1998; **143:** 2045–2056.
43. Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci USA* 1998; **95:** 5672–5677.