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# 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.[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)-$\beta$1 respectively induced erythroid differentiation, neuronal formation and myogenesis of ES cells.[11–13] To date, however, there has been no report that growth factors efficiently and specifically induced mouse ES cells to become cardiomyocytes except for one recent report, which investigated TGF-$\beta$ 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-$\beta$,[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.

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*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 $\mu$mol/L 2-mercaptoethanol (2-ME), 1 $\mu$mol/L sodium pyruvate, 0.1 $\mu$mol/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.*

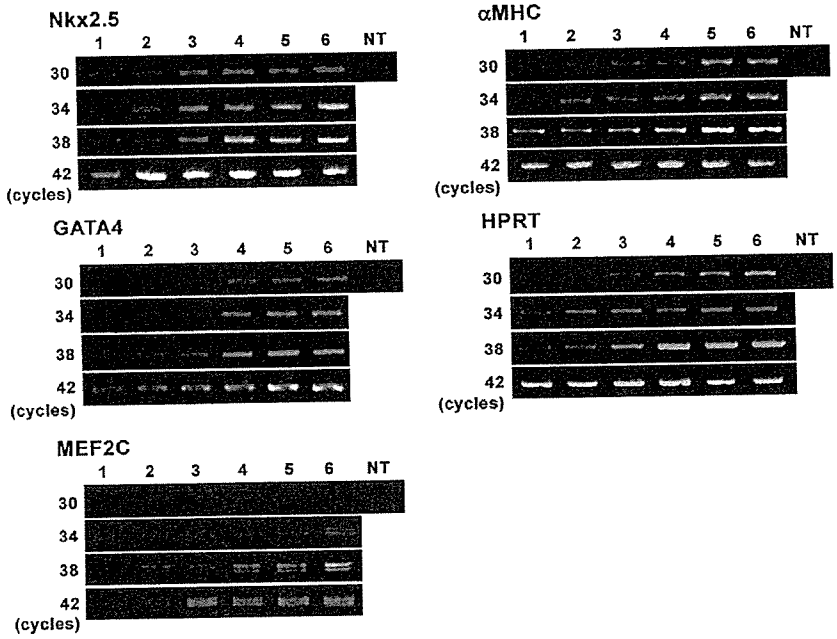


Fig 1. Optimization of the semi-quantitative RT-PCR assay. Serially diluted plasmid DNA containing Nkx2.5 cDNA (0.08, 0.15, 0.31, 0.63, 1.25 and 2.5 pg DNA in lanes 1, 2, 3, 4, 5 and 6, respectively) was used for control samples for semi-quantitative RT-PCR of Nkx2.5. Total RNA extracted from adult mouse 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

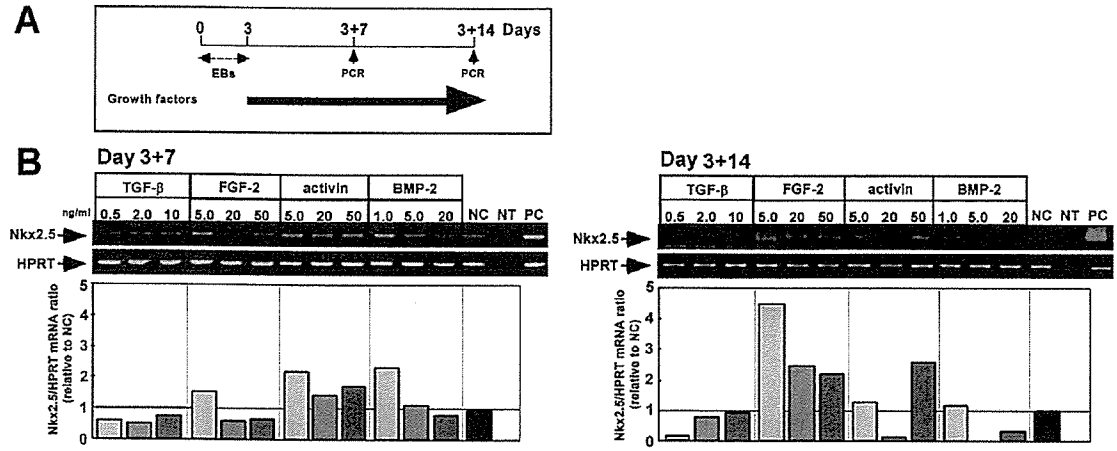


Fig 2. Nkx2.5 mRNA expression in ES cells treated with one of the 4 growth factors. (A) Experimental schedule. ES cells were cultured in suspension for 3 days to form EBs, and subsequently plated and cultured with media containing one of 4 recombinant growth factors at various concentrations for an additional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

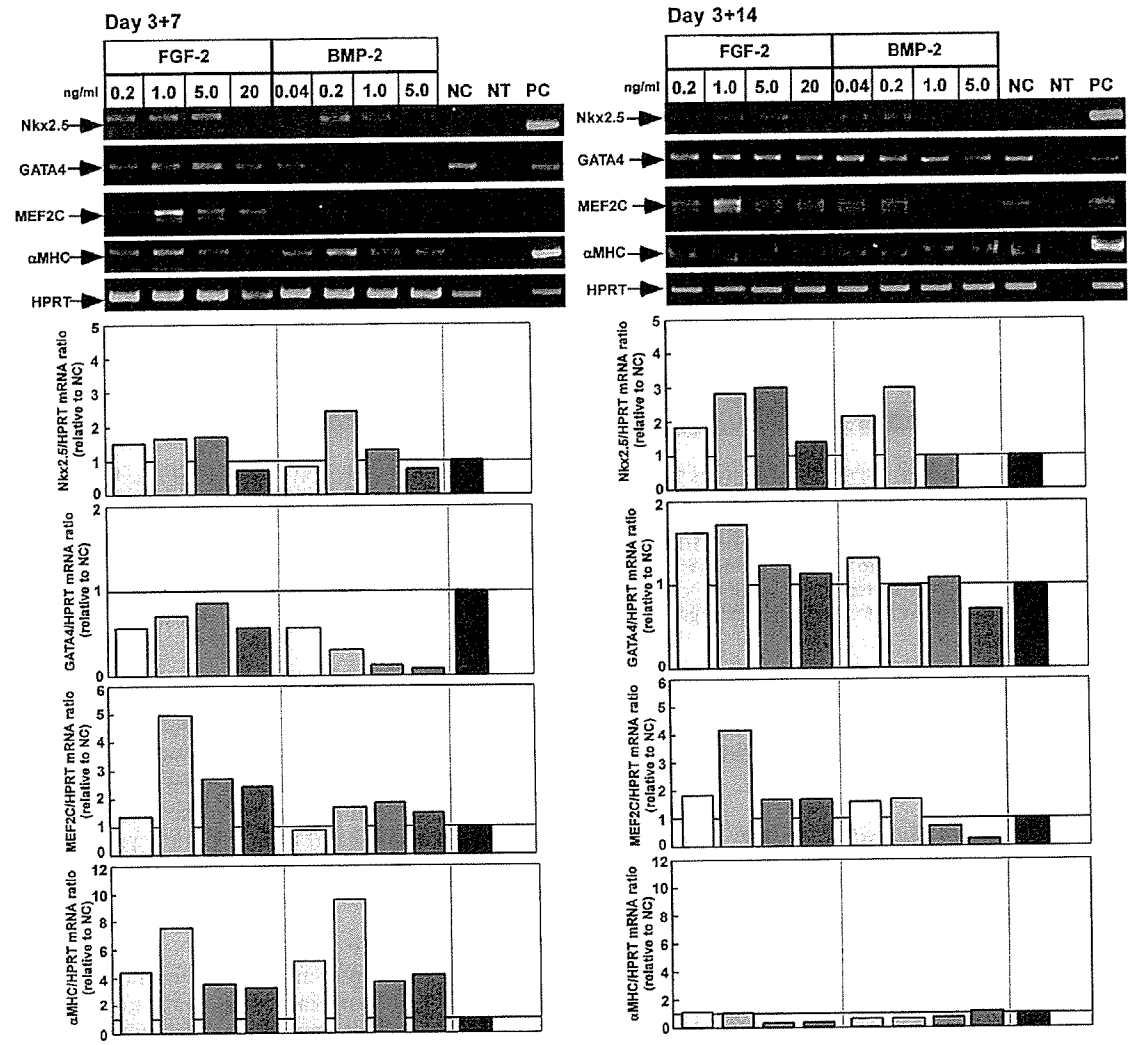


Fig 3. Effects of various doses of FGF-2 or BMP-2 on in vitro cardiomyogenic differentiation of ES cells. ES cells were cultured with FGF-2 or BMP-2 at various concentrations on the same schedule as shown in Fig 2A. RT-PCR analysis of cardiomyocyte-specific genes, Nkx2.5, GATA4, MEF2C and αMHC, was performed. 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

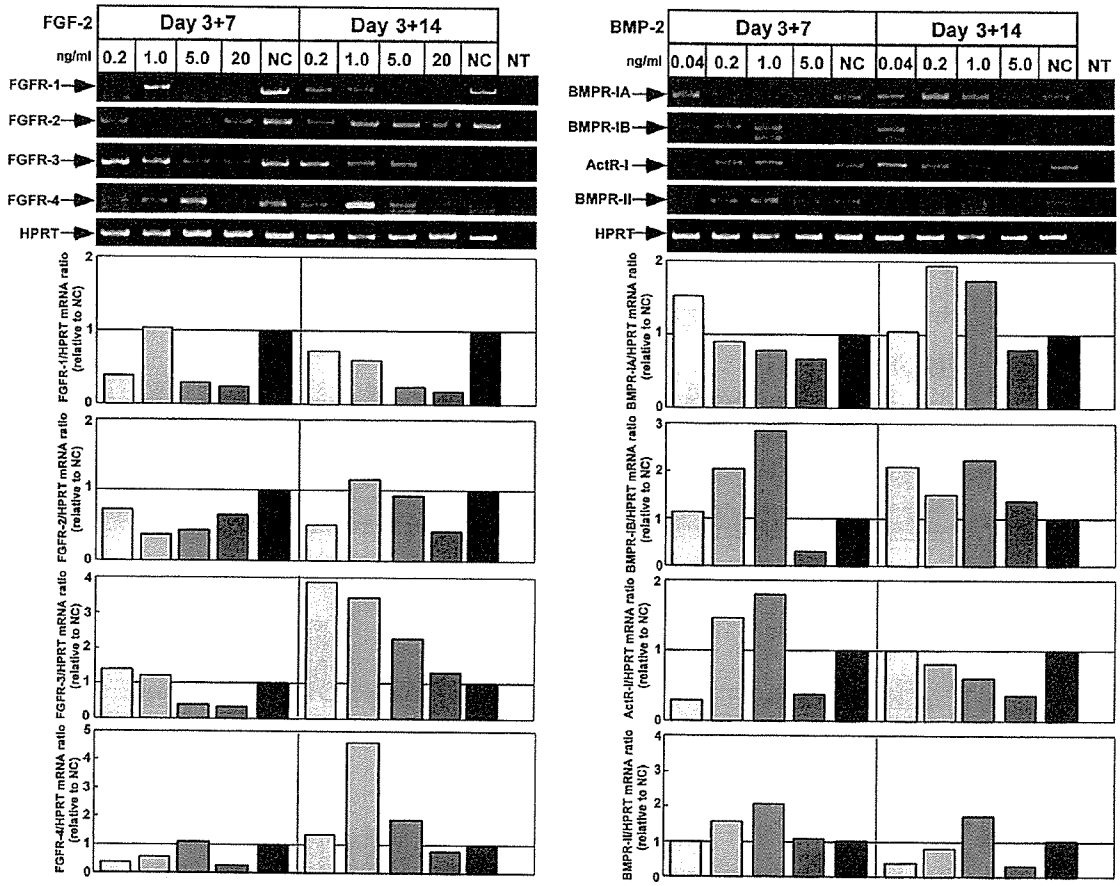


Fig 4. Effects of various doses of FGF-2 or BMP-2 on the expression levels of FGFRs and BMPRs in ES cells. Experimental schedule was the same as shown in Fig 2A. RT-PCR analyses of FGFRs (FGFR-1, FGFR-2. FGFR-3 and FGFR-4) and BMPRs (BMPR-IA, BMPR-IB, ActR-I and BMPR-II), and the calculation of FGFR/HPRT 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 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 (0.2–20 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 αMHC 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 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 Fig 3, 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 (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 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

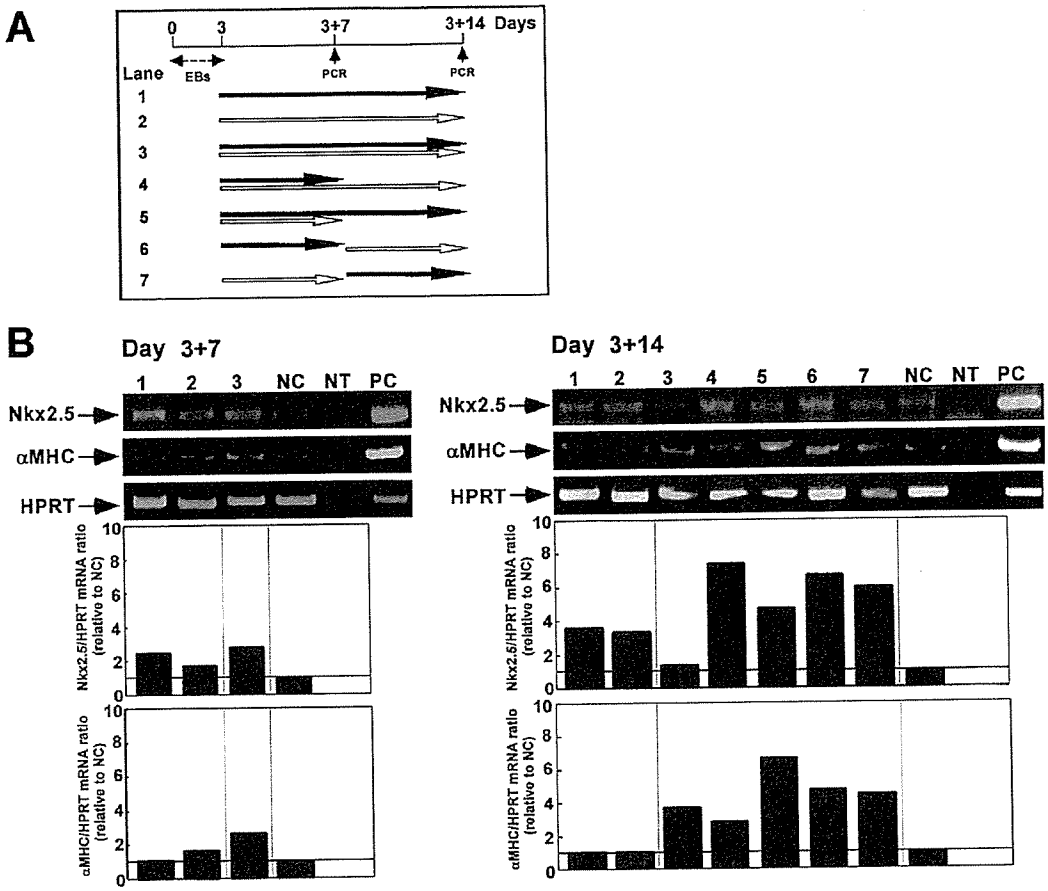


Fig 5. Effects of the combination of FGF-2 and BMP-2 on in vitro cardiomyogenic differentiation of ES cells. (A) Experimental schedules. ES cells were cultured with FGF-2 (1.0 ng/ml) and BMP-2 (0.2 ng/ml) following different schedules. Black and white arrows represent the addition of FGF-2 and BMP-2,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

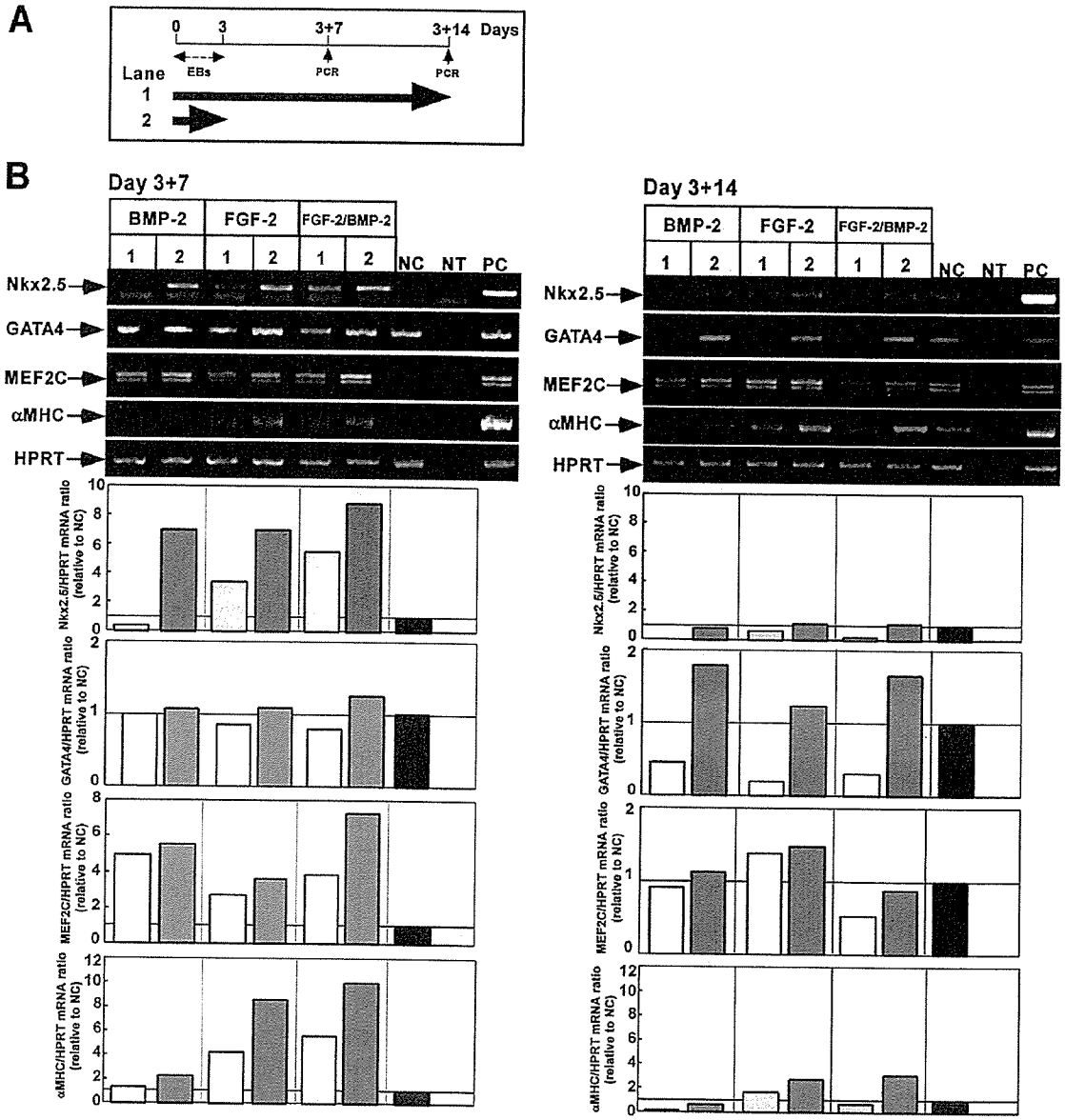


Fig 6. Efficacy of FGF-2 and BMP-2 for early cardiomyogenic differentiation. (A) Experimental schedules. In vitro differentiation was initiated by the formation of EBs without LIF in the same way as already shown, and FGF-2 (1.0 ng/ml) and/or BMP-2 (0.2 ng/ml) were added to the culture media either during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

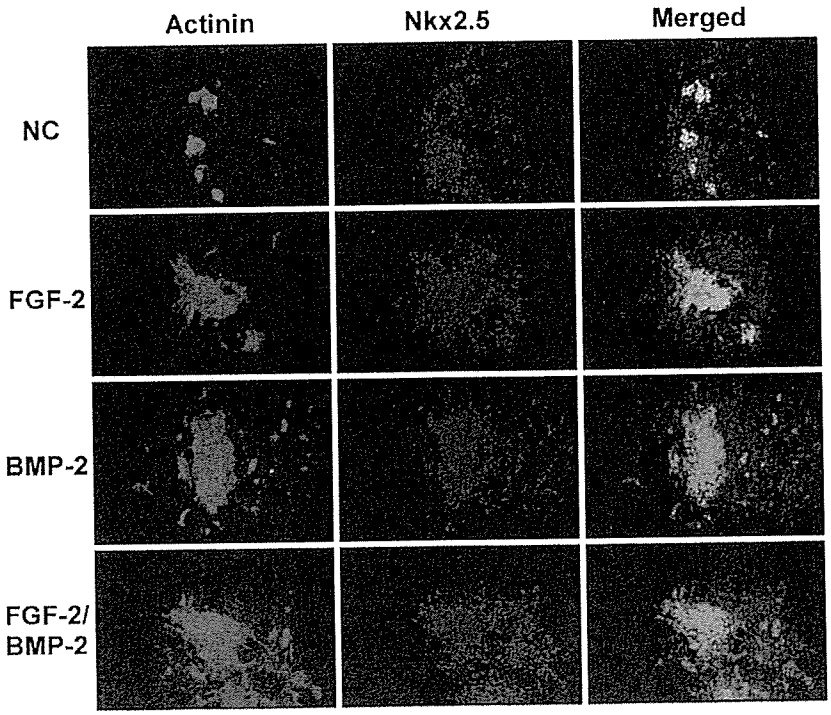


Fig 7. Immunocytochemical staining of cardiomyocyte-specific genes, actinin and Nkx2.5. FGF-2 (1.0 ng/ml) and/or BMP-2 (0.2 ng/ml) were added for 7 days after the formation of EBs using the same protocol as shown in Fig 5A. ES cells on day 3+7 were immunocytochemically stained with anti-actinin and anti-Nkx2.5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

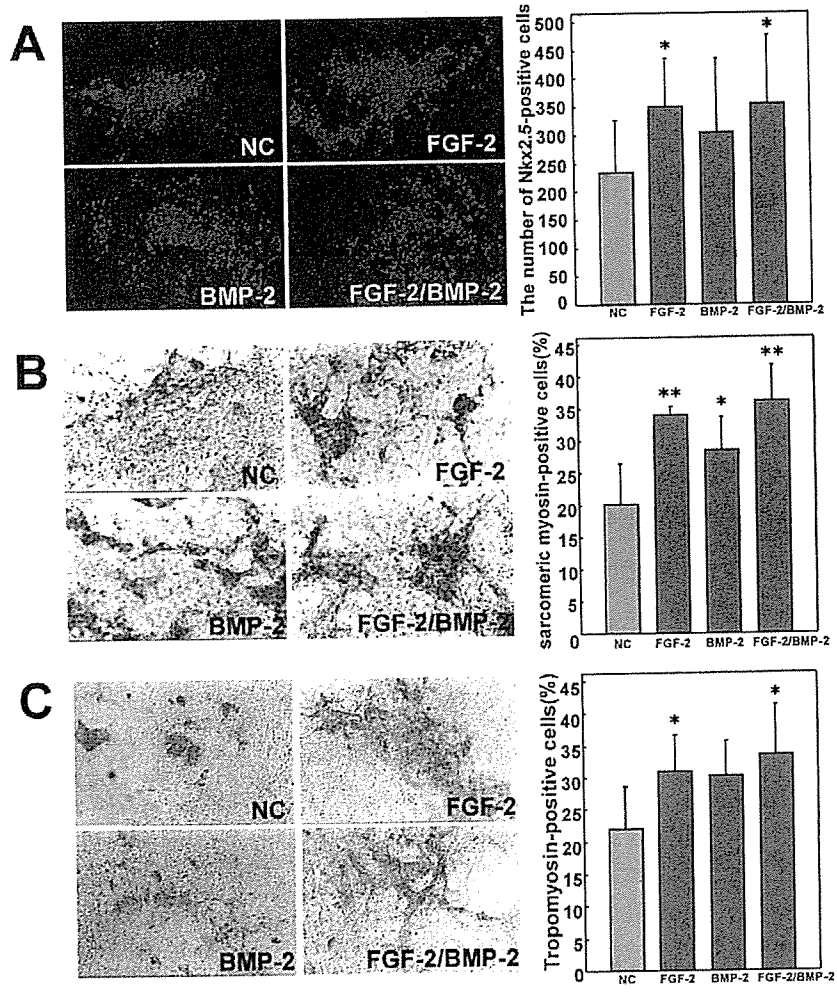


Fig 8. Immunohistochemical and quantitative analysis of ES cell-derived cardiomyocytes. ES cells were cultured with FGF-2 (1.0 ng/ml) and/or BMP-2 (0.2 ng/ml) under the same protocol as shown in Fig 5A. ES cells on day 3+7 were immunocytochemically stained with an antibody of anti-Nkx2.5 (A), anti-sarcomeric 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, ×100). *p<0.05; **p<0.001.

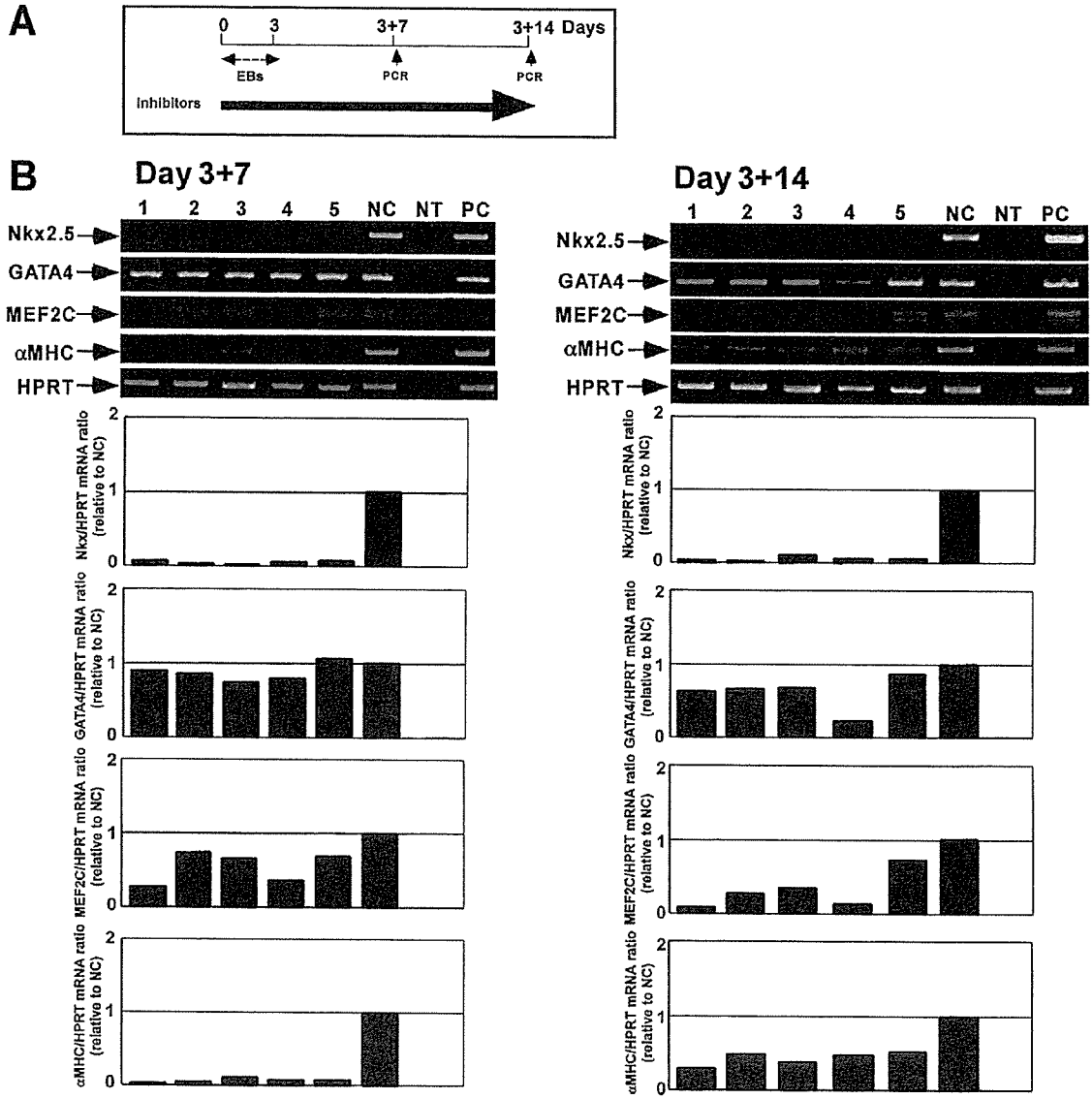


Fig 9. Inhibition of FGF-2 and BMP-2 attenuated in vitro cardiomyogenic differentiation of ES cells. (A) Experimental schedule. In vitro differentiation was initiated by the formation of EBs without LIF in the same way as already shown. Either anti-FGF-2 antibody (2.5 μg/ml; lane 1), BMPR-1B/Fc (1.0 μg/ml; lane 2), Noggin/Fc chimera (0.1 μg/ml; lane 3), anti-TGF-β antibody (1.5 μg/ml; lane 4) or anti-PDGF antibody (1.5 μg/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-$\beta$ or anti-PDGF antibody was used in the same way as an experimental control because it has been already shown that TGF-$\beta$ 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 $\alpha$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 $\alpha$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 $\alpha$-actin, $\alpha$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 $\alpha$MHC, 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, $\alpha$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-$\beta$ 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-$\beta$ (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-$\beta$ (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-$\beta$ 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-$\beta$ 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? 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.

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ORIGINAL PAPER

# Tumor suppressor WARTS ensures genomic integrity by regulating both mitotic progression and $G_1$ tetraploidy checkpoint function

Shin-ichi Iida[1,2], Toru Hirota[1], Tetsuro Morisaki[1], Tomotoshi Marumoto[1], Toshihiro Hara[1], Shinji Kuninaka[1], Shinobu Honda[1], Ken-ichiro Kosai[3], Michio Kawasuji[2], David C Pallas[4] and Hideyuki Saya*,[1]

[1]*Department of Tumor Genetics and Biology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan;* [2]*Department of Cardiovascular Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan;* [3]*Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases Kurume University, Kurume 830-0011, Japan;* [4]*Department of Biochemistry and Winship Cancer Center, Emory University School of Medicine, Atlanta, GA 30322, USA*

**Defects in chromosomes or mitotic spindles activate the spindle checkpoint, resulting in cell cycle arrest at prometaphase. The prolonged activation of spindle checkpoint generally leads to mitotic exit without segregation after a transient mitotic arrest and the consequent formation of tetraploid $G_1$ cells. These tetraploid cells are usually blocked to enter the subsequent S phase by the activation of p53/pRb pathway, which is referred to as the $G_1$ tetraploidy checkpoint. A human homologue of the *Drosophila* warts tumor suppressor, WARTS, is an evolutionarily conserved serine–threonine kinase and implicated in development of human tumors. We previously showed that WARTS plays a crucial role in controlling mitotic progression by forming a regulatory complex with zyxin, a regulator of actin filament assembly, on mitotic apparatus. However, when WARTS is activated during cell cycle and how the loss of WARTS function leads to tumorigenesis have not been elucidated. Here we show that WARTS is activated during mitosis in mammalian cells, and that overexpression of a kinase-inactive WARTS in Rat1 fibroblasts significantly induced mitotic delay. This delay resulted from prolonged activation of the spindle assembly checkpoint and was frequently followed by mitotic slippage and the development of tetraploidy. The resulting tetraploid cells then abrogated the $G_1$ tetraploidy checkpoint and entered S phase to achieve a DNA content of 8N. This impairment of $G_1$ tetraploidy checkpoint was caused as a consequence of failure to induce p53 expression by expressing a kinase-inactive WARTS. WARTS thus plays a critical role in maintenance of ploidy through its actions in both mitotic progression and the $G_1$ tetraploidy checkpoint.**
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**Keywords:** cell cycle; mitotic kinase; spindle checkpoint; p53; polyploid

*Correspondence: H Saya; E-mail: hsaya@gpo.kumamoto-u.ac.jp
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## Introduction

Mitosis is precisely regulated by sequential biochemical reactions, including protein phosphorylation and degradation, in order to achieve the equal separation of replicated sister chromatids into the two daughter cells. Checkpoints exist at various stages of the cell cycle to prevent improper progression of cells through the cycle and thereby to preserve the integrity of the genome. During mitotic phase, the spindle assembly checkpoint plays an important role in ensuring that chromosomes align correctly at the metaphase plate in preparation for their segregation. Defects in chromosomes or the mitotic spindle at this stage result in activation of the spindle assembly checkpoint and cell cycle arrest at prometaphase. Prolonged activation of this checkpoint leads to the skipping of chromosome segregation and cytokinesis and the consequent formation of tetraploid cells. The replication of DNA in tetraploid cells that have entered the subsequent $G_1$ phase is usually blocked by p53- and pRb-dependent cell cycle arrest, which is referred to as the $G_1$ tetraploidy checkpoint (Margolis *et al.*, 2003). Impairment of this checkpoint allows the tetraploid cells to maintain their proliferative potential, a process known as tetraploidization. Mitotic dysfunction alone is thus not sufficient to induce and maintain tetraploidization, which is considered a frequent precursor of aneuploidy during tumorigenesis (Shackney *et al.*, 1989).

The *warts* gene (also referred to as *lats*) was identified on the basis of its ability to act as a tumor suppressor in *Drosophila melanogaster* (Justice *et al.*, 1995). The protein encoded by this gene possesses a serine–threonine kinase catalytic domain that is highly similar to those of members of the human myotonic dystrophy protein kinase family. Kinases of this family have been shown to contribute to various mitotic events (Toyn and Johnston, 1994; Yasui *et al.*, 1998). A human homolog of warts, termed WARTS (or LATS1), has been identified (Nishiyama *et al.*, 1999; Tao *et al.*, 1999), and mice deficient in the corresponding protein develop malignant tumors similarly to the analogous *Drosophila* mutant (St John *et al.*, 1999). Furthermore, a missense

point mutation of *WARTS* was recently identified in human soft-tissue sarcoma, suggesting that alterations in WARTS function might be of pathological importance in human tumorigenesis (Hisaoka *et al*., 2002).

Immunolocalization studies have shown that human WARTS is found in cytoplasm and the centrosome in interphase cells, that it localizes to the mitotic apparatus, including the spindle poles and mitotic spindle, during metaphase–anaphase, and that it is present at the midbody during telophase (Nishiyama *et al*., 1999). Moreover, WARTS is phosphorylated during mitosis and interacts with zyxin, a regulator of actin filament assembly, at the mitotic apparatus, and this mitosis-specific interaction plays an important role in the control of mitotic progression (Hirota *et al*., 2000). WARTS is thus a key player in mitotic events in mammalian cells, and loss of its function disrupts normal mitotic regulation, possibly leading to chromosomal instability and tumor development. However, given that the kinase activity of WARTS in cells has not been unambiguously detected to date, the mechanisms by which WARTS contributes to cell cycle events have remained largely unknown.

To provide insight into WARTS function, we have now examined the kinase activity of this protein during the cell cycle in mammalian cells. WARTS was activated specifically during mitosis, with peak activity apparent at the prometaphase/metaphase transition. Overexpression of a kinase-inactive WARTS mutant in Rat1 fibroblasts induced a pronounced delay in entry into anaphase as well as subsequent mitotic slippage and the development of tetraploidy. Furthermore, such tetraploid cells did not arrest at $G_1$ phase and entered another round of DNA synthesis, resulting in tetraploidization. In contrast, Rat1 cells overexpressing wild-type WARTS underwent cell death when the spindle assembly checkpoint was continuously activated by nocodazole treatment. Our results suggest that the kinase activity of WARTS is required for proper mitotic progression, for cell death in response to prolonged activation of the spindle assembly checkpoint, and for activation of the $G_1$ tetraploidy checkpoint. The loss of WARTS function might thus induce mitotic aberration followed by tetraploidization, which leads to the consequent development of aneuploidy during tumorigenesis.

## Results

### *Mitotic activation of WARTS kinase activity*

To investigate possible changes in the kinase activity of human WARTS during the cell cycle, we first performed *in vitro* kinase assays with WARTS immunoprecipitated from asynchronous or mitotic HeLa cells. Whereas, autophosphorylation of WARTS *in vitro* was almost undetectable with the protein isolated from asynchronous cells, it was markedly increased with WARTS derived from mitotic cells (Figure 1a). To exclude the possibility that phosphorylation of WARTS detected in this assay was due to the presence of another kinase in the immunoprecipitates, we generated stable transfectants of Rat1 fibroblasts that constitutively express wild-type or kinase-inactive forms of WARTS tagged at their $NH_2$-termini with the Myc epitope (WTW1 and KDW1 cells, respectively) (Figure 1b). The kinase-inactive WARTS mutant (K734A) was constructed by replacing lysine 734 with alanine. This conserved lysine residue has been proven to play a key role in the recognition of the phosphate group of Mg-ATP (Hanks *et al*., 1988), and changing this lysine residue reportedly resulted in a kinase-inactive protein (Xia *et al*., 2002). Both exogenous WARTS proteins localized to centrosomes and the cytoplasm during interphase and to the mitotic apparatus during mitosis (Figure 1c), consistent with the cell cycle-dependent localization of the endogenous protein as previously reported (Nishiyama *et al*., 1999).

To examine whether the kinase activity of wild-type Myc-WARTS was increased during mitosis like that of endogenous WARTS, we synchronized WTW1 cells at prometaphase by treatment with nocodazole and then released them into normal medium. Myc-WARTS was immunoprecipitated from cell lysates at various times thereafter with antibodies to Myc and was subjected to the *in vitro* kinase assay. A protein that migrated at the position of Myc-WARTS was phosphorylated during mitotic progression, with the maximal activity apparent 10–15 min after release from prometaphase arrest (Figure 1d). However, no phosphorylated protein of the same size was detected when the same experiment was performed with KDW1 cells (Figure 1e). Furthermore, Myc-WARTS immunoprecipitated from asynchronous WTW1 cells exhibited only a low level of autophosphorylation activity (Figure 1e). These findings indicated that WARTS is autophosphorylated, rather than phosphorylated by other kinases interacting with WARTS, during mitosis and that WARTS is thus activated specifically during M phase.

### *Chromosomal instability induced by overexpression of kinase-inactive WARTS*

We next investigated the effects of constitutive expression of the wild-type and mutant WARTS proteins on the phenotype of Rat1 cells. The rate of proliferation was reduced in WTW1 cells and, to an even greater extent, in KDW1 cells compared with that apparent in parental Rat1 cells (Figure 2a). Flow cytometric analysis of WTW1 cells revealed two prominent peaks of cells in $G_1$ (2N) and in $G_2$–M (4N), similar to the cell cycle distribution of the parental cells (Figure 2b). In contrast, the proportion of 2N cells was reduced and that of 4N and >4N cells was increased for KDW1 cells compared with parental cells. These observations were supported by microscopic analysis of cells stained with propidium iodide to reveal DNA; nuclei of KDW1 cells thus tended to be much larger than those of WTW1 and parental Rat1 cells (Figure 2c). In addition, KDW1 cells frequently manifested lagging chromosomes during mitosis as well as enlarged, multilobular, or multiple nuclei (data not shown).

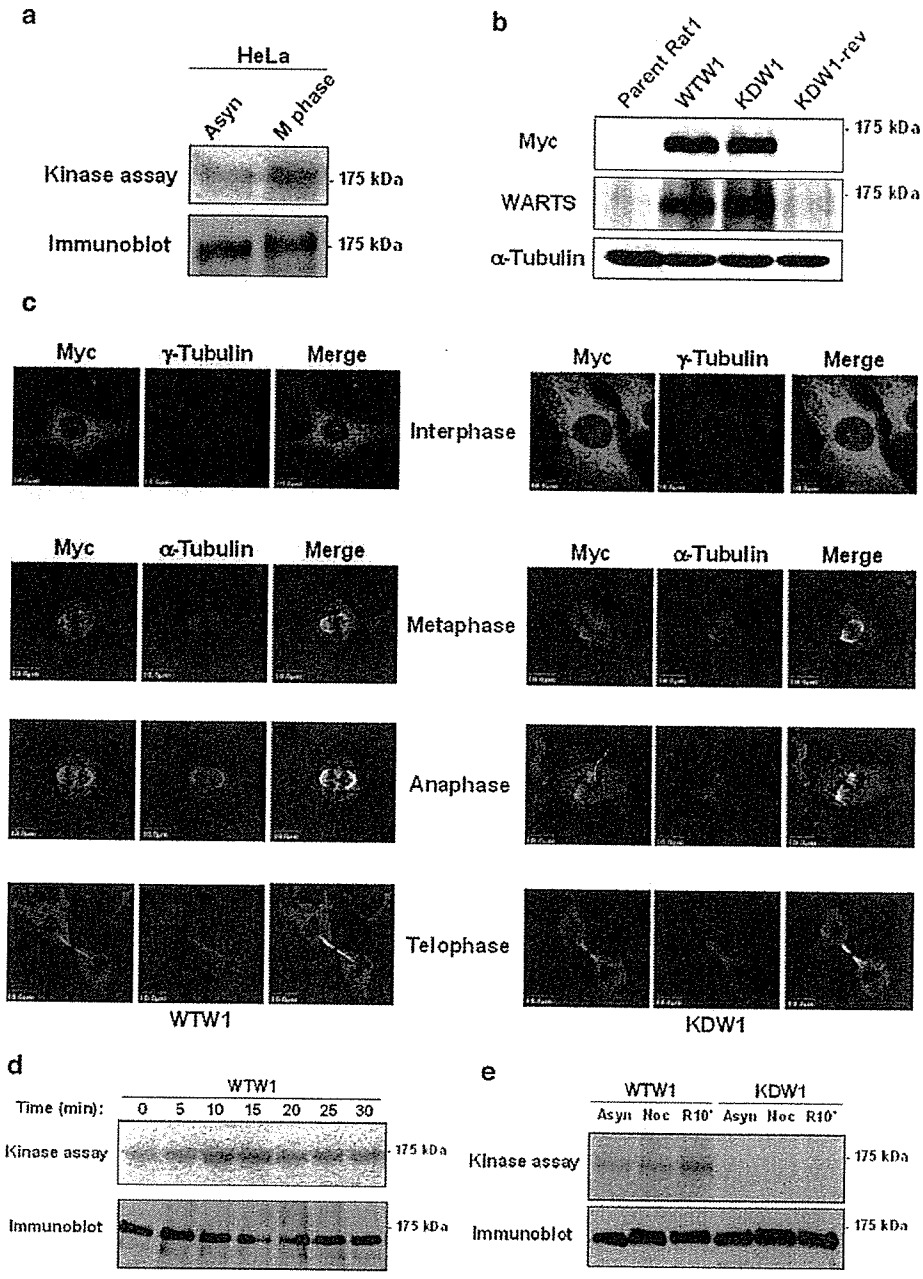


**Figure 1** Mitosis-specific activation of WARTS. (**a**) Autophosphorylation of WARTS kinase during mitosis. Asynchronous (Asyn) or mitotic HeLa cells were lysed and subjected to immunoprecipitation with antibodies to WARTS, and the resulting precipitates were subjected either to immunoblot analysis with the same antibodies (bottom panel) or to an *in vitro* kinase assay with [$\gamma$-$^{32}$P]ATP (top panel). (**b**) Immunoblot analysis of Myc-WARTS expression. Total lysates of parental Rat1, WTW1, KDW1, and KDW1-rev cells were subjected to SDS–PAGE on a 6% gel followed by immunoblot analysis with antibodies to Myc (9E10), to WARTS (Hirota *et al*., 2000), or to $\alpha$-tubulin (B-5-1-2) as a loading control. (**c**) Subcellular localization of Myc-WARTS during the cell cycle. WTW1 and KDW1 cells were fixed and processed for indirect immunofluorescence staining. Cells at interphase were immunostained with antibodies to Myc (FITC, green) and to $\gamma$-tubulin (Alexa 568, red); cells at metaphase, anaphase, or telophase were immunostained with antibodies to Myc (FITC, green) and to $\alpha$-tubulin (Alexa 568, red). (**d**) Activation of wild-type Myc-WARTS during mitosis. WTW1 cells were treated with nocodazole for 10 h and then released into normal medium for the indicated times. Cell lysates were then subjected to immunoprecipitation with antibodies to Myc, and the resulting precipitates were subjected to the *in vitro* kinase assay with [$\gamma$-$^{32}$P]ATP. The same amounts of immunoprecipitates were then subjected to immunoblot analysis with antibodies to WARTS. (**e**) WTW1 or KDW1 cells were treated with nocodazole for 10 h and then released into normal medium for 0 (Noc) or 10 (R10′) min before analysis as in (**d**). Asynchronous cells were similarly analysed

To confirm the increased ploidy of KDW1 cells, we counted the chromosomes of the three cell types. The distribution of chromosome number for KDW1 cells differed markedly from that for WTW1 or parental Rat1 cell populations (Figure 2d). Of 50 metaphase KDW1 cells scored, 15 cells (30%) appeared almost tetraploid, whereas all parental Rat1 cells or WTW1 cells scored were diploid. The fact that intermediate

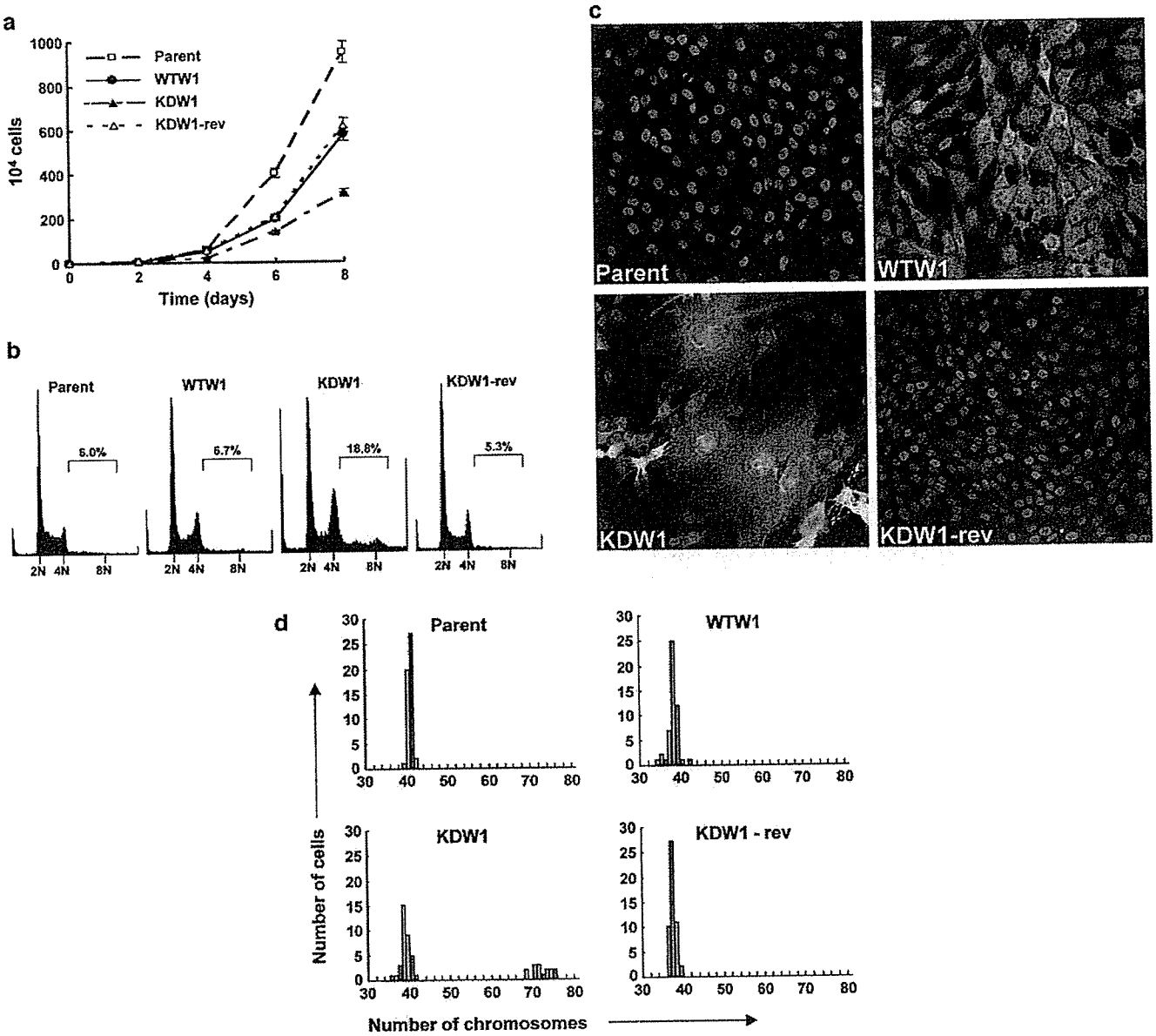


**Figure 2** Polyploidy in Rat1 cells induced by overexpression of kinase-inactive WARTS. (**a**) Cell proliferation was analysed by seeding $1 \times 10^4$ cells (parental Rat1, WTW1, KDW1, or KDW1-rev) in complete medium into 10-cm dishes and determining the cell number every 48 h. Data are means$\pm$s.e.m. of values from three independent experiments. (**b**) Flow cytometric analysis of asynchronous cell cultures. Cells were harvested with trypsin-EDTA, fixed with 70% ethanol, treated with RNase A (100 U/ml), and stained with propidium iodide (50 µg/ml). Percentages represent the number of cells with a DNA content of >4N. (**c**) Nuclear morphology of parental Rat1, WTW1, KDW1, and KDW1-rev cells. Cells were fixed and processed for indirect immunofluorescence staining with antibodies to Myc (FITC, green) and for staining of DNA with propidium iodide (red). (**d**) Distribution of chromosome number was determined for 50 metaphase cells of the four cell lines

levels of ploidy were not observed in KDW1 cells suggested that the tetraploidy of these cells might result from either failure of chromosome segregation or impairment of cytokinesis.

At later passages (>23 doublings), KDW1 cells are somewhat unstable, generally containing some cells (~10% of the total population) that have lost the expression of the kinase-inactive form of WARTS protein (Figures 1b and 2c). We cloned these revertant KDW1 cells (termed KDW1-rev cells) and showed that they were phenotypically similar to WTW1 or parental Rat1 cells (Figure 2). The expression of the kinase-inactive WARTS protein thus appeared to be directly associated with the polyploid phenotype of KDW1 cells, suggesting that the kinase activity of WARTS is required for maintenance of ploidy.

### *Prolonged activation of the spindle assembly checkpoint induced by overexpression of kinase-inactive WARTS*

To define more precisely the effect of the kinase-inactive WARTS on mitotic progression, we determined the

duration of mitosis in parental Rat1, WTW1, KDW1, and KDW1-rev cells with the use of time-lapse differential interference contrast microscopy. More than 90% of parental Rat1 or WTW1 cells executed mitosis within 30 min, whereas only 4% of KDW1 cells exited mitosis within this time (Figure 3a). In KDW1-rev cells, lost of the kinase-inactive WARTS expression significantly increased the number of cells (54%) exiting mitosis within 30 min. Most KDW1 cells with a prolonged duration of mitosis (>100 min) exited mitosis without undergoing cytokinesis. Expression of the kinase-inactive WARTS mutant thus interfered with normal mitotic progression and frequently induced skipping mitosis followed by polyploidization. These phenotypes might contribute to the reduced growth rate of KDW1 cells in comparison to WTW1 or KDW1-rev cells (Figure 2a).

To examine the nature of the mitotic delay in cells expressing the WARTS mutant, we synchronized WTW1 and KDW1 cells at prometaphase by nocodazole treatment, released them into normal medium, and then determined their mitotic distribution at various times thereafter by immunostaining with antibodies to α-tubulin and staining of DNA with propidium iodide. Whereas WTW1 cells began to enter anaphase after 15 min and had reached telophase by 25 min after release, most KDW1 cells remained in metaphase 25 min after release (Figure 3b). Quantitative analysis confirmed that KDW1 cells manifested a marked delay in anaphase entry compared with WTW1 cells (Figure 3c).

Delay in anaphase entry is usually caused either by activation of the spindle assembly checkpoint or by disruption of proteolysis by the anaphase-promoting complex or cyclosome (APC/C) (King *et al*., 1995; Tugendreich *et al*., 1995; Amon, 1999). To investigate the former possibility, we examined recruitment of MAD2 protein to kinetochores, which is thought to occur specifically at unattached kinetochores during prometaphase and is required for maintenance of spindle assembly checkpoint signaling (Shah and

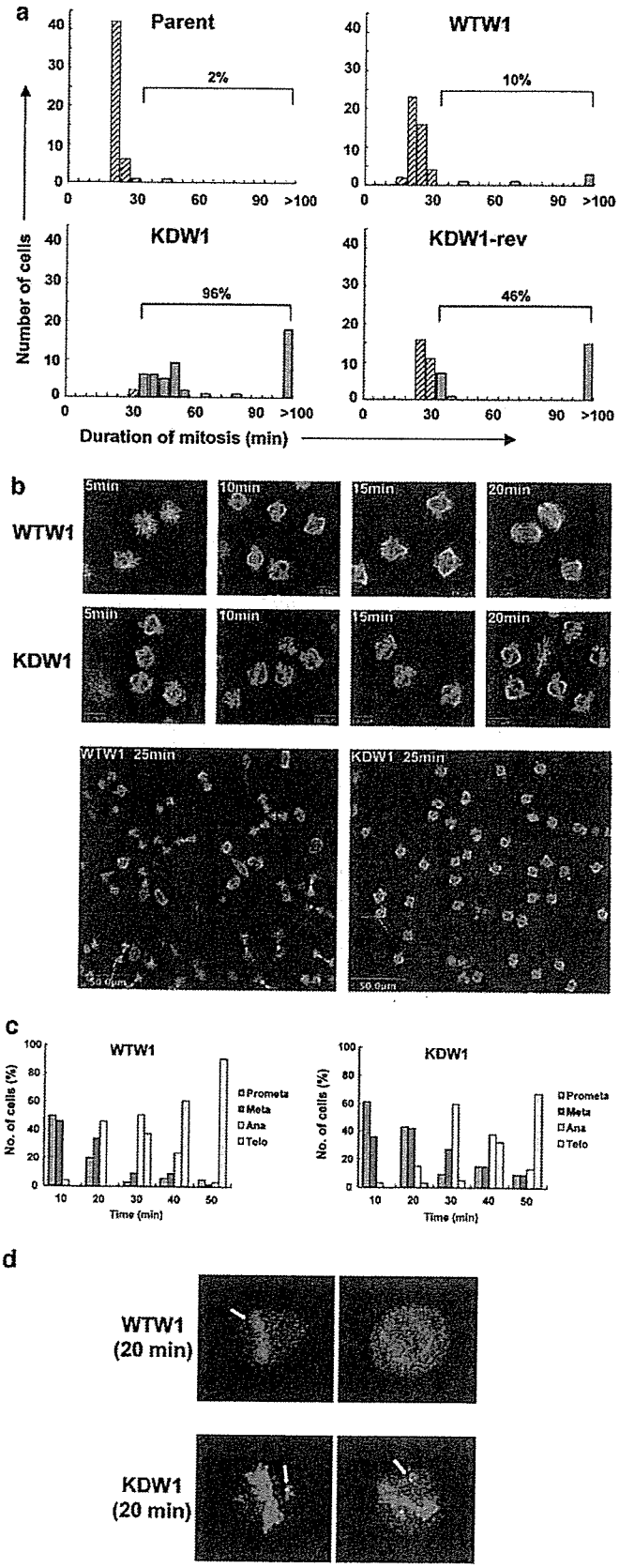


**Figure 3** Mitotic delay due to activation of the spindle assembly checkpoint in cells overexpressing kinase-inactive WARTS. (**a**) Quantitation of the time required for individual parental Rat1, WTW1, KDW1, and KDW1-rev cells to complete mitosis. The duration of mitosis was determined morphologically by time-lapse videomicroscopy; its onset was defined as the time when the cell first became rounded and refractile and its end was defined as the time when the cell flattened back onto the dish. A total of 50 cells were analysed for each cell line. Percentages represent the number of cells that required >30 min to execute mitosis. (**b**) Characterization of mitotic progression in WTW1 and KDW1 cells. Cells were treated with nocodazole for 10 h and then released into normal medium for the indicated times. They were then fixed and processed for indirect immunofluorescence staining with antibodies to α-tubulin (FITC, green) and for staining of DNA with propidium iodide (red). (**c**) Quantitation of mitotic progression. WTW1 and KDW1 cells were treated and analysed as in (**b**), and the percentage of cells in prometaphase, metaphase, anaphase, or telophase at the indicated times after release from nocodazole treatment was determined from examination of 200–300 cells of each line. (**d**) Immunostaining with antibodies to MAD2. WTW1 and KDW1 cells were treated with nocodazole, released into normal medium for 20 min, and then subjected to indirect immunostaining with antibodies to MAD2 (FITC, green) and to staining of DNA with propidium iodide (red). Representative cells are shown. Arrows indicate kinetochores positive for MAD2

Cleveland, 2000). At 20 min after release from nocodazole treatment, most WTW1 cells had already entered anaphase; all chromosomes were aligned at the metaphase plate and exhibited only faint staining for MAD2 (Figure 3d). In contrast, in most KDW1 cells at this time, some of the chromosomes had still not aligned at the metaphase plate and MAD2 staining was pronounced at the kinetochores of such misaligned chromosomes. Given that a single MAD2-positive unaligned chromosome is sufficient to activate the spindle assembly checkpoint (Nicklas, 1997), our observations suggest that prolonged activation of this checkpoint contributes to the mitotic delay observed in KDW1 cells. The kinase-inactive WARTS mutant may thus interfere with kinetochore function or assembly of the mitotic spindle, resulting in prolonged activation of the spindle assembly checkpoint and prometaphase delay. WARTS localizes to the mitotic spindle during mitotic phase (Nishiyama *et al*., 1999), and the peak of WARTS kinase activity coincided with the timing of prometaphase-to-metaphase progression (Figure 1d). These data suggest that activation of WARTS might play an important role in the regulation of events related to the spindle attachment to kinetochore and chromosome alignment.

### *DNA synthesis without cell division after prolonged activation of the spindle assembly checkpoint in KDW1 cells*

Progression of tumors to a highly aneuploid state is thought to occur in at least two discrete steps. First, failure of the primary control of chromosome segregation or cytokinesis results in the generation of a tetraploid cell. Second, failure of a p53- and pRb-dependent surveillance mechanism that normally arrests such tetraploid cells in $G_1$ leads to aberrant DNA synthesis (Margolis *et al*., 2003). We have shown that prolonged activation of the spindle assembly checkpoint appears to be responsible for the pronounced mitotic delay in KDW1 cells and for allowing such cells to exit mitosis without undergoing cytokinesis, resulting in the formation of $G_1$ cells with a DNA content of 4N. We, therefore next determined whether the $G_1$ tetraploidy checkpoint prevents these cells from undergoing another round of DNA synthesis. To this end, we investigated the fate of cells treated with nocodazole to induce prolonged activation of the spindle assembly checkpoint. Asynchronous cells were treated with nocodazole for 12 h, and mitotic cells were then collected by the 'shake off' procedure and transferred to new dishes for further treatment with nocodazole. The cells that flattened back onto the culture dishes in the presence of nocodazole were analysed. The percentage of these cells with a DNA content of 8N was markedly greater for the KDW1 line than for the WTW1 line (Figure 4a), indicating that a substantial proportion of KDW1 cells with a DNA content of 4N underwent an additional round of DNA synthesis to yield a DNA content of 8N. Most KDW1-rev cells appeared to arrest with a DNA content of 4N. On the other hand, nocodazole-treated WTW1 cells showed a marked reduction in the proportion of cells with a DNA content of 4N that was concomitant with an increase in the size of the sub-$G_1$ population, indicating that a large number of WTW1 cells with a 4N DNA content underwent cell death as a result of the prolonged treatment with nocodazole. These results suggest that, in cells that have become tetraploid as a result of mitotic errors, overexpression of kinase-inactive WARTS induces abrogation of the $G_1$ tetraploidy checkpoint and that overexpression of wild-type WARTS induces cell death rather than $G_1$ arrest.

### *Role of WARTS in p53 induction at the $G_1$ tetraploidy checkpoint*

To investigate why KDW1 cells with a DNA content of 4N fail to undergo $G_1$ arrest, we examined the expression of p53, a key player in activation of the $G_1$ tetraploidy checkpoint (Minn *et al*., 1996; Lanni and Jacks, 1998; Andreassen *et al*., 2001). Although p53 was accumulated in KDW1-rev cells treated with nocodazole for 26 h as described above, its abundance was markedly reduced in similarly treated KDW1 cells (Figure 4b). It was not possible to examine WTW1 cells in this experiment because most of these cells underwent apoptosis in response to prolonged nocodazole treatment as shown above (Figure 4a). These results suggest that abrogation of the $G_1$ tetraploidy checkpoint in KDW1 cells is due to a defect in the induction of p53. Overexpression of kinase-inactive WARTS thus induces not only activation of the spindle assembly checkpoint but also abrogation of the $G_1$ tetraploidy checkpoint, resulting in the development of polyploidy. It therefore appears that the kinase activity of WARTS plays important roles in both mitotic progression and the $G_1$ tetraploidy checkpoint.

## Discussion

This study is the first demonstration that WARTS kinase is activated at the prometaphase/metaphase transition. Our data suggest that WARTS activation plays an important role in the regulation of spindle attachment to kinetochore and chromosome alignment. Thus, impairment of WARTS function leads to the activation of spindle assembly checkpoint. Although the function of the spindle assembly checkpoint is thought to be to ensure the correct assembly of a functioning mitotic spindle before exit from mitosis, the ability of cells to arrest in mitosis varies (Kung *et al*., 1990); many cell types thus undergo only a transient mitotic arrest and then exit mitosis without chromosome segregation. Consistent with these observations, we have shown here that cells expressing a kinase-inactive WARTS, KDW1 cells, frequently experience prolonged activation of the spindle assembly checkpoint and subsequently exit mitosis without cell division and therefore became tetraploid. Cells that have evaded mitotic arrest after activation of the spindle assembly checkpoint by treatment with an inhibitor of microtubule assembly have previously been shown to undergo p53-dependent

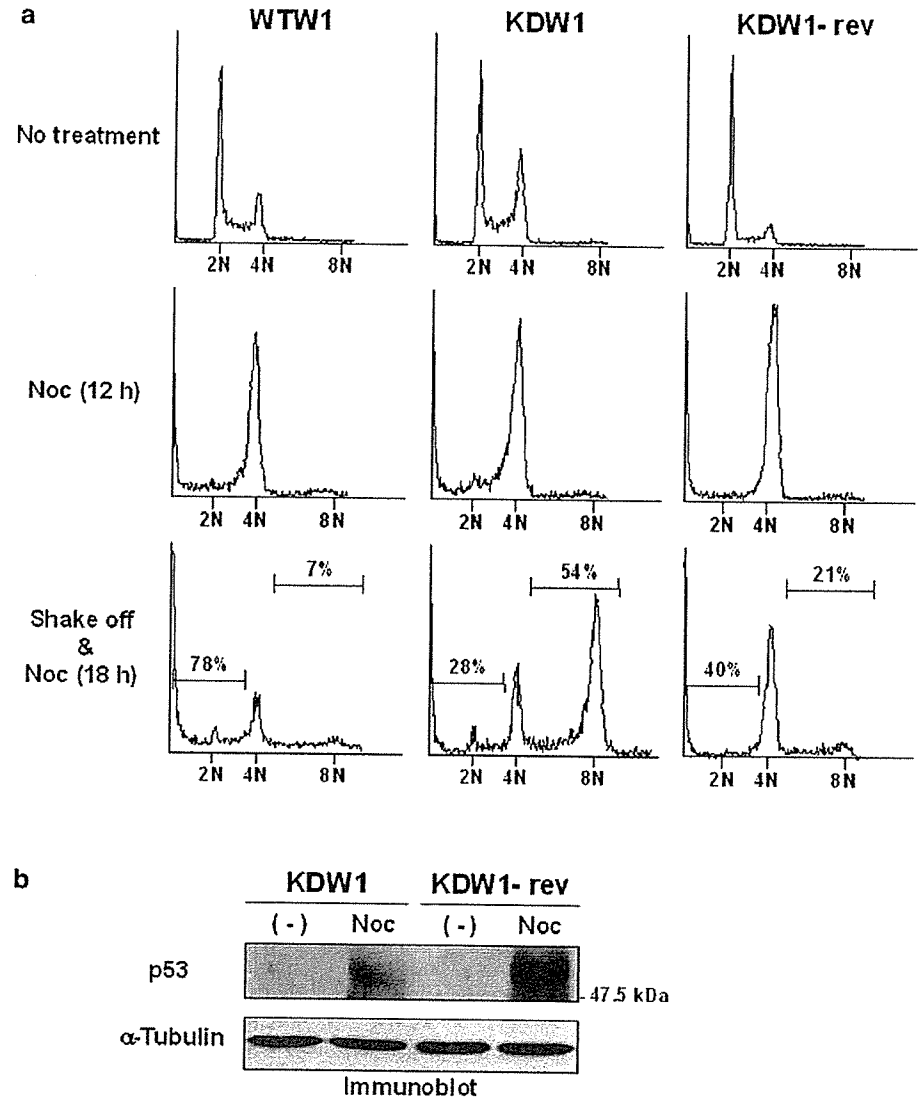


**Figure 4** Role of WARTS in the $G_1$ tetraploidy checkpoint. (**a**) Asynchronous WTW1, KDW1, or KDW1-rev cells (upper panels) were treated with nocodazole for 12 h (middle panels), and mitotic cells were then collected by the shake-off procedure and transferred to new dishes for incubation for an additional 18 h with nocodazole (lower panels). The DNA content of attached cells was determined by flow cytometry. Percentages refer to the number of cells with a DNA content of $<4N$ or $>4N$. (**b**) KDW1 and KDW1-rev cells were treated (or not) with nocodazole for 26 h as described in (**a**), and attached $G_1$ cells were then collected and lysed. Lysates were subjected to SDS–PAGE on a 10% gel followed by immunoblot analysis with antibodies to p53 (R-19, Santa Cruz) or to α-tubulin

$G_1$ arrest (Minn *et al.*, 1996; Lanni and Jacks, 1998). Moreover, cells treated with an inhibitor of actin assembly to induce failure of cytokinesis underwent p53-dependent $G_1$ arrest even when formation of the mitotic spindle and chromosome segregation proceeded normally, suggesting that tetraploidy *per se* leads to $G_1$ arrest (Andreassen *et al.*, 2001). Our detection of a substantial population of KDW1 cells with a DNA content of 8N after prolonged activation of the spindle assembly checkpoint suggests that overexpression of the kinase-inactive WARTS mutant impaired $G_1$ tetraploidy checkpoint function. In fact, we further demonstrated that the induction of p53 was defective in nocodazole-treated KDW1 cells, indicating that the kinase activity of WARTS is required for p53 induction. This defect of p53 induction appears to be responsible for failure to activate the $G_1$ tetraploidy checkpoint and entry into subsequent S phase in KDW1 cells. We are currently investigating how the activated WARTS induces p53 accumulation when mitosis is failed.

Since the only one clone of kinase-inactive mutant was available in this study, it raises the possibility that overexpression of kinase-inactive WARTS might cause some additional effects in this clone other than simply interfering the function of the endogenous WARTS expressed in Rat 1 cells. However, the distribution of mutant WARTS is essentially the same as that of wild-type WARTS (Figure 1c), suggesting that mutants WARTS does not associate with some unexpected targets. Furthermore, KDW1-rev cells, which lost the mutant WARTS expression, showed the similar phenotypes to the parental Rat 1 cells. It is thus likely that the kinase-inactive WARTS inhibits endogenous WARTS in a dominant-negative manner.

It was previously reported that ectopic expression of WARTS in human cancer cells induces upregulation of BAX proteins and leads to apoptosis (Yang *et al.*, 2001; Xia *et al.*, 2002). In contrast, our study shows that overexpression of wild-type WARTS in Rat1 fibroblasts resulted in neither apoptosis nor cell cycle arrest. These discrepancies might be attributed to differences between cancer cells and normal cells. It should be noted that overexpression of wild-type WARTS in Rat1 fibroblasts (WDW1 cells) preferentially induced apoptosis when spindle damage was induced by nocodazole treatment, whereas KDW1-rev cells did not (Figure 4a). These differences in sensitivity of the two cell lines to nocodazole may be due to the differences in the expression levels of wild-type WARTS protein. These findings suggest that overactivation of WARTS kinase induces apoptosis in the presence of tumorigenic signals and cytotoxic stress such as spindle damage or DNA damage. Therefore, activation and/or overexpression of WARTS may provide a potential specific therapeutic approach for treating malignant tumors.

Overexpression of other mitotic kinases (Polo-like kinase 1, Aurora-A, Aurora-B) also induces mitotic failure concomitant with multinucleation and an increase in centrosome number, and the absence of p53-dependent checkpoint function exacerbates this phenotype (Meraldi *et al.*, 2002). In this regard, it thus appears that WARTS is unique in that it regulates not only mitotic progression but also p53-mediated checkpoint function. Inactivation of WARTS kinase alone thus possibly leads to genomic instability and development of tumors through mitotic failure, $G_1$ checkpoint abrogation, and the survival of abnormal cells.

## Materials and methods

### *Cell culture and synchronization*

Parental Rat1 cells and stable transfectants were cultured in DME-F12 medium supplemented with 10% fetal bovine serum without antibiotics. For cell synchronization in prometaphase, cells were incubated for 10 h with nocodazole (50 ng/ml) and then released into normal medium.

### *Generation of Rat1 cells stably expressing WARTS*

Rat1 cells in six-well plates were transfected, with the use of FuGENE 6 (Roche), with the expression vector pUHG10-3 containing the cDNA for either wild-type or kinase-inactive forms of human WARTS. The kinase-inactive WARTS mutant (K734A) was constructed by replacing lysine 734 in the ATP-binding site with alanine. After 48 h, the cells were cultured in the presence of hygromycin B (0.2 mg/ml) (Wako), and antibiotic-resistant cell colonies were isolated 1 week later by ring cloning.

### *Immunofluorescence microscopy*

Cells were grown in 35-mm dishes to ~70% confluence. Fixation and permeabilization were performed as previously described (Hirota *et al.*, 2000). After washing with PBS, the cells were incubated with primary antibodies including mouse monoclonal antibodies to Myc (9E10, Roche), mouse monoclonal antibodies to α-tubulin (B-5-1-2, Sigma), rabbit polyclonal antibodies to MAD2 (Marumoto *et al.*, 2003), rat polyclonal antibodies to α-tubulin (Novus Biologicals) or to γ-tubulin (Sigma). Immune complexes were detected with fluorescein isothiocyanate (FITC) — conjugated antibodies to mouse immunoglobulin G (IgG), FITC-conjugated antibodies to rabbit IgG (Amersham Pharmacia), or Alexa 568-conjugated antibodies to rat IgG (Molecular Probes). The stained cells were mounted and observed as previously described (Hirota *et al.*, 2000).

### *Immune-complex kinase assay*

Cells were lysed on ice for 20 min in a solution containing 0.5% NP-40, 100 mM NaCl, 2 mM EDTA, 5 mM EGTA, 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM KCl, 100 μM leupeptin, 1 μM pepstatin, 100 μM Tosyl Lysine Chloromethyl Ketone (TLCK), 100 nM okadaic acid, 2 mM benzamidine, and 1 mM dithiothreitol. The lysate was centrifuged at 18000 *g* for 20 min, and portions of the supernatant (0.2–2.0 mg/ml) were incubated at 4°C first for 2 h with antibodies to Myc (9E10) and then, after the addition of protein G-Sepharose beads (Amersham Pharmacia), for an additional 90 min. The beads were then isolated by centrifugation, washed with cell lysis buffer, and subjected to the *in vitro* kinase assay. Kinase reactions were performed for 30 min at 30°C in a final volume of 30 μl containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 μCi of [γ-$^{32}$P]ATP (3000 Ci/mmol) (Amersham Pharmacia), and 1 μM microcystin. The phosphorylated proteins were resolved by SDS–PAGE on a 5–20% acrylamide-gradient gel and visualized by autoradiography.

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