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Ratio of Wnt3a to BMP4 doses is critical to their synergistic effects on proliferation of differentiating mouse embryonic stem cells

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Abstract. *Objectives*: To investigate potential interactions between bone morphogenetic protein (BMP) and Wnt signalling on differentiating mouse embryonic stem cells (mESC). *Materials and methods*: Mouse embryonic stem cells were cultured with differing combinations of Wnt3a, BMP4 and inhibitors of Wnt, BMP, PI-3K (phosphoinositide 3-kinase), p38, ERK1/2 (extracellular signal-regulated kinase 1/2) and JNK (c-Jun N-terminal kinase) pathways. *Results*: We found that Wnt3a synergized with BMP4 to promote mESC proliferation. Furthermore, the relative ratio of Wnt3a to BMP4 doses was critical to their synergistic effects, which could be abolished by using Dkk-1, noggin or the inhibitors of PI-3K, p38, ERK1/2 and JNK pathways. We also demonstrated that combination of Wnt3a and BMP4 could suppress ectodermal differentiation of mESCs. Moreover, inhibitors of PI-3K, p38, ERK1/2 and JNK pathways could negate this effect. *Conclusion*: Relative ratio of Wnt3a to BMP4 doses is critical to their synergistic effect on differentiating mESC proliferation, which may work through PI-3K, p38, ERK1/2 and JNK pathways.

INTRODUCTION

Mouse embryonic stem cell (mESC) lines were first established by culturing the inner cell mass of blastocyst cells on feeder layers (Evans & Kaufman 1981; Martin 1981). Embryonic stem cells (ESC) have since then been demonstrated to be pluripotent, by their ability to differentiate in culture into ectodermal, mesodermal and endodermal derivatives. The uses of mESCs to generate chimaeras and transgenic mice further proved pluripotency of ESCs. Although much has been learned concerning the biology of ESCs, many questions regarding their *in vitro* expansion remain elusive.

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Bone morphogenetic protein (BMP) and Wnt (a combination of the names of Wg, wingless, and Int genes) families of signalling proteins have been implicated in multiple developmental events (Nakayama *et al.* 2000; Nusse 2005). This has led to much interest in studying their actions on ESCs because their differentiation may be considered as a diminutive scope of developmental processes. BMPs belong to the transforming growth factor-β (TGF-β) superfamily, and over 20 BMP family members have been identified. Like other members of the TGF-β superfamily, BMPs exert their actions through types I and II serine/threonine kinase receptor complex. Three mammalian type I BMP receptors, ALK-2 (activin receptor-like kinase-2), BMPR-IA (BMP type IA receptor)/ALK-3 and BMPR-IB/ALK-6 (Koenig *et al.* 1994; ten Dijke *et al.* 1994), and three BMP type II receptors, BMPR-II, ActR-II (activin type II receptor) and ActR-IIB, have been recognized (Liu *et al.* 1995; Rosenzweig *et al.* 1995; Yamashita *et al.* 1995). Gene ablation experiments have shown that BMPs are pivotal to mouse development as well as to functions of cardiovascular, pulmonary, nervous, reproductive and urogenital systems in adulthood (Goumans & Mummery 2000). In mESCs, BMP4 is able to maintain the pluripotency of mESCs in serum-free conditions in cooperation with LIF (leukaemia inhibiting factor) (Ying *et al.* 2003). Recent studies have demonstrated that the BMP signal could repress neural differentiation in both embryos (Wilson & Edlund 2001) and ESCs (Tropepe *et al.* 2001; Ying *et al.* 2003), as well as induce lineage fates of mesoderm, endoderm and trophoblast (Nakayama *et al.* 2000; Li *et al.* 2001; Xu *et al.* 2002; Ying *et al.* 2003; Pera *et al.* 2004). In contrast, LIF is able to inhibit mesoderm and endoderm differentiation but facilitates differentiation into neural lineage (Ying *et al.* 2003).

There are at least 19 Wnt family members. Wnt proteins (Moon *et al.* 2004) exert their actions on target cells by binding to the Fz (Frizzled)/LDL (low-density lipoprotein)/LRP (receptor-related protein) complex at the cell surface. These receptors then transduce the signal to several intracellular components that include Dsh (Dishevelled), GSK-3 (glycogen synthase kinase-3β), axin, APC (adenomatous polyposis coli) and β-catenin. Cytoplasmic β-catenin levels are constitutively low through continuous proteasome-mediated degradation, which is controlled by a complex containing GSK-3/APC/axin. Degradation of β-catenin is blocked when Wnt signals are transduced. Subsequently, β-catenin accumulates in the cytoplasm and nucleus, which then interacts with transcription factors such as LEF/TCF (lymphoid enhancer-binding factor 1/T cell-specific transcription factor) to modulate transcription of the target genes. *Wnt3* gene ablation inhibits formation of primitive streak and consequently, mesoderm and definitive endoderm (Liu *et al.* 1999). These phenotypes have been substantiated in models of β-catenin gene ablation, showing failure of primitive streak formation and additional defects (Huelsken *et al.* 2000; Lickert *et al.* 2002; Morkel *et al.* 2003). Recent data have indicated the involvement of Wnt signalling in maintenance of pluripotency of ESCs (Sato *et al.* 2004) by proving that the use of 6-bromoindirubin-3′-oxime (BIO), a specific pharmacological inhibitor of GSK-3, was able to retain the undifferentiated phenotype and expression of pluripotent state-specific transcription factors Oct-3/4, Rex-1 and Nanog in ESCs.

Interactions between BMP and Wnt signalling differ in organogenesis or the adult functions of varied systems (Jin *et al.* 2001; Fischer *et al.* 2002; Theil *et al.* 2002; He *et al.* 2004; Kleber *et al.* 2005; Mbalaviele *et al.* 2005; Winkler *et al.* 2005) (Table 1). BMP and Wnt signalling could antagonize each other for self-renewal or differentiation of stem cells at specific tissues and times. Experimental conditional disruption of BMPR-IA function has indicated that BMP signalling counteracted Wnt signalling to repress intestinal stem cell self-renewal (He *et al.* 2004). Likewise, BMP4 and Wnt3a acted in an opposite manner in specification of melanocyte lineage from the trunk neural crest (Jin *et al.* 2001). In contrast, BMP and Wnt signalling can also exert their actions synergistically. For example, BMP and Wnt signalling act in concert in

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Table 1. The potential interactions between Wnt and BMP signalling on stem cells (literature review)

Interaction	Reference
BMP and Wnt signalling act in concert to regulate the Emx2 transcription in governing the pattern formation of dorsal telencephalon in mice.	Theil *et al*. 2002
BMP and Wnt signalling cooperate in the control of formation of the apical ectodermal ridge and the dorsal–ventral axis in the limb.	Soshnikova *et al*. 2003
An important role of the interactions between BMP and Wnt signalling in tumour suppression.	Nishanian *et al*. 2004
BMP signalling counteracted Wnt signalling to repress intestinal stem cell self-renewal.	He *et al*. 2004
BMP4 and Wnt3a acted in an opposite manner on specification of the melanocyte lineage from the trunk neural crest.	Jin *et al*. 2001
The synergism between β-catenin and BMP2 in promoting differentiation and new bone formation.	Mbalaviele *et al*. 2005
In terms of differentiation and self-renewal of neural crest stem cells, BMP signalling antagonizes the action of Wnt signalling on the sensory fate induction; on the other hand, BMP and Wnt signalling cooperate to facilitate the maintenance of the multipotency of neural crest stem cells.	Kleber *et al*. 2005

the regulation of Emx2 expression in pattern formation of the dorsal telencephalon (Theil *et al*. 2002). Recent evidence has also demonstrated synergism between β-catenin and BMP2 in promoting differentiation and new bone formation (Mbalaviele *et al*. 2005). Interestingly, in terms of differentiation and self-renewal of neural crest stem cells, BMP signalling antagonizes action of Wnt signalling on sensory cell fate induction, yet on the other hand, BMP and Wnt signalling cooperate to facilitate maintenance of multipotency of neural crest stem cells (Kleber *et al*. 2005).

Bone morphogenetic protein and Wnt signalling have both been confirmed to play important roles in pattern formation during embryogenesis as well as in proliferation and differentiation during postnatal life (Kishigami & Mishina 2005; Reya & Clevers 2005; Zhang & Li 2005; Marikawa 2006; van Amerongen & Berns 2006). Hence, we aimed to investigate potential interactions between BMP and Wnt signalling on differentiating mESCs. We found that Wnt3a synergized with BMP4 to promote proliferation of differentiating mESCs. Furthermore, the relative ratio of Wnt3a to BMP4 doses was critical to their synergistic effects, which may work through PI-3 K (phosphoinositide 3-kinase), p38, ERK1/2 (extracellular signal-regulated kinase 1/2) and JNK (c-Jun N-terminal kinase) pathways.

MATERIALS AND METHODS

Cell cultures

Mouse embryonic stem cells (from ATCC SCRC-1003 mice) were maintained on mitomycin C-treated STO feeder cells (from ATCC CRL-1503) at 37 °C, under 5% CO_2 in air and in the presence of 1000 units/mL mouse leukaemia inhibitory factor (LIF) (Chemicon, Temecula, CA, USA). The mESC medium contained Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine and 4.5 g/L glucose (Invitrogen, Carlsbad, California, USA), supplemented

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with 10% foetal bovine serum (Hyclone, Logan, UT, USA), 15 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), 1.2 g/L sodium bicarbonate, 1% penicillin and streptomycin, 0.025 mg/mL ampicillin, and 0.1 mM 2-mercaptoethanol (all from Sigma, St. Louis, MO, USA). For most of the experiments in the study, to avoid influences from feeder cells and LIF, mESCs were grown on gelatin-coated dishes (0.1% gelatin; Sigma), with normal mESC medium in the absence of LIF and feeder cells. For the long-term culture to determine effects of cell proliferation or differentiation, cell culture media were changed every other day, that is, on days 0, 2 and 4, and relevant assessments were performed on days 1, 3 and 5.

Growth factors and inhibitors

In the study, growth factors and inhibitors used included BMP4 (R&D systems, Minneapolis, MN, USA), WNT3a (R&D systems), noggin (R&D systems), Dkk-1 (Dickkopf-1) (R&D systems), LY294002 (Sigma), SB202190 (Sigma), U0126 (Sigma) and SP600125 (Sigma). Whenever inhibitor activity was required for experimental procedures, cells were exposed to them 2 h before any growth factor was added to the culture medium.

Cell proliferation assays

To analyse cell growth curves, 9.6×10^3 cells were seeded in 12-well plates coated with 0.1% gelatin. Triplicate plates were installed for each treatment. At defined times, cell viability was then examined by trypan blue exclusion assays, and duplicate cell counts were performed for each plate. In addition, 5-bromo-2′-deoxyuridine (BrdU) incorporation assay (Roche, Mannheim, Germany; cat. no. 11 647 229 001) was also used to assess mESC proliferation as described in the instructional manual. Briefly, BrdU labelling solution (final concentration: 10 μM BrdU) was added to the medium and cells were re-incubated for an additional 4 h at 37 °C. After removing labelling solution, FixDenat solution was added and cells were incubated at 25 °C for 30 min FixDenat solution was then removed and cells were incubated with anti-BrdU-POD working solution at 25 °C for 90 min. After removing anti-BrdU-POD working solution and rinsing specimens three times with washing solution, substrate solution was added at 25 °C and the sample was incubated for 5 min. With 1 M H_2SO_4 as a stop solution, absorbance of samples was measured in an ELISA reader at 450 nm (reference wavelength: 690 nm).

Western blot and antibody analysis

five to twenty micrograms of each cell lysate were fractionated by 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. Primary antibodies used in the study included anti-p-Smad1 (Cell Signalling Technologies, Beverly, MA, USA, #9511), anti-p-GSK3 (Cell Signalling Technologies, #9336), anti-Oct-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-5279), anti-alkaline phosphatase (Santa Cruz Biotechnology, sc-5279) and anti-β-actin (Chemicon, MAB1501). We used goat antirabbit or goat antimouse secondary antibodies, conjugated to horseradish peroxidase (Dako, Carpinteria, CA, USA) and developed blots using ECL (Amersham Biosciences, Buckinghamshire, UK).

Reverse transcription-polymerase chain reaction

Total RNA extraction for cultured cells was performed by using RNeasy Mini Kit (Qiagen, Valencia, CA, USA).

First-strand cDNA was synthesized by reverse transcription (RT) (SuperScript II; Invitrogen). The polymerase chain reaction(PCR) was then performed using primers for: BMPR-IA (forward [f], CTTCTCCAGCTGCTTTTGCT; reverse [r], CGCCATTTACCCATCCATAC), BMPR-IB (f, GTACTGCAGGGCCACAATTT; r, TCTTCCAGGCTCAGGTGACT), BMPR-II

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(f, GGGAGCACGTGTTATGGTCT; r, CAGAAACTGATGCCAAAGCA), Fzd5 (f, ACATGG-AACGATTCCGCTAC; r, GGCCATGCCAAAGAAATAGA), Fzd7 (f, TTTCAAGAGGAGGC-CAAGAA; r, GGCTTTGCCTGTAAAAGCTG), Pax6 (f, AGACTTTAACCAAGGGCGGT; r, TAGCCAGGTTGCGAAGAACT), Brachyury (f, TCCCGAGACCCAGTTCATAG; r, ATT-GTCCGCATAGGTTGGAG), HNF-4 (f, GAGGTCCATGGTGTTTAAGGAC; r, CTGCAG-CAGGTTGTCAATCTTGG), Stella (f, TTCCGAGCTAGCTTTTGAGG; r, TTTCAGCAC-CGACAACAAAG) and β-actin (f, CCCACACTGTGCCCATCTAC; r, AGTACTTGCGCT-CAGGAGGA). PCR conditions were set up as follows: 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and a final cycle of 5 min at 72 °C.

Statistical analysis

All experiments were performed in triplicate. SPSS version 11.0 (SPSS Inc., Chicago, IL, USA) was the statistical software used to undertake the statistical analyses. Comparisons between multiple numeric data sets were performed using one-way ANOVA followed by Tukey's honestly significant difference *post hoc* test. Differences between groups were deemed significant where $P < 0.05$. The statistical graphs were plotted using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

BMP and Wnt signalling in mESCs

To be sure that BMP and Wnt signalling occurs in mESCs, initially we checked whether there was mRNA expression of BMP and Wnt receptors in them. First, total RNA was extracted from mESCs that had been grown on gelatin with appropriate mESC culture medium and LIF, until displaying about 80% confluence in the culture dish. Using RT-PCR on total RNA from the cells, we found mRNA transcripts for BMP receptors, such as BMPR-IA (BMP receptor IA), BMPR-IB and BMPR-II, and Wnt receptors, such as Frizzled 5 (Fzd5) and Frizzled 7 (Fzd7) (Fig. 1a). Subsequently, BMP and Wnt signal transduction were checked in cells that had already been withdrawn from LIF for 16 h. BMP4 100 ng/mL can activate its signal pathway after 1 h of incubation, demonstrated by increased levels of phospho-Smad1 (p-Smad1) (Fig. 1b). In addition, we found that Wnt3a 100 ng/mL can activate the Wnt canonical pathway after 1 h, or more obviously 2 h, of incubation, verified by increased levels of phospho-GSK3 (p-GSK3) in Western blot analysis (Fig. 1c).

BMP4 synergizes with Wnt3a to promote proliferation of differentiating mESCs

As BMP and Wnt signalling have been reported to have different interactions on different types of stem cells (Reya & Clevers 2005; Zhang & Li 2005), we were interested in whether BMP4 and Wnt3a exert their actions on mESCs synergistically, antagonistically or independently. To avoid LIF influence on the effects of BMP4 and Wnt3a, mESCs were grown on gelatin with regular mESC culture media, but in the absence of LIF. First, four kinds of treatment, BMP4 100 ng/mL, Wnt3a 100 ng/mL, BMP4 100 ng/mL + Wnt3a 100 ng/mL and control (no growth factors), were added into the culture medium. Effects of these treatments on proliferation of mESCs were assessed continuously for 6 days by cell counting. Interestingly, the combination of BMP4 (100 ng/mL) and Wnt3a (100 ng/mL) significantly enhanced proliferation of differentiating mESCs on day 5 and day 6 compared to control, BMP4 or Wnt3a alone groups (Fig. 2).

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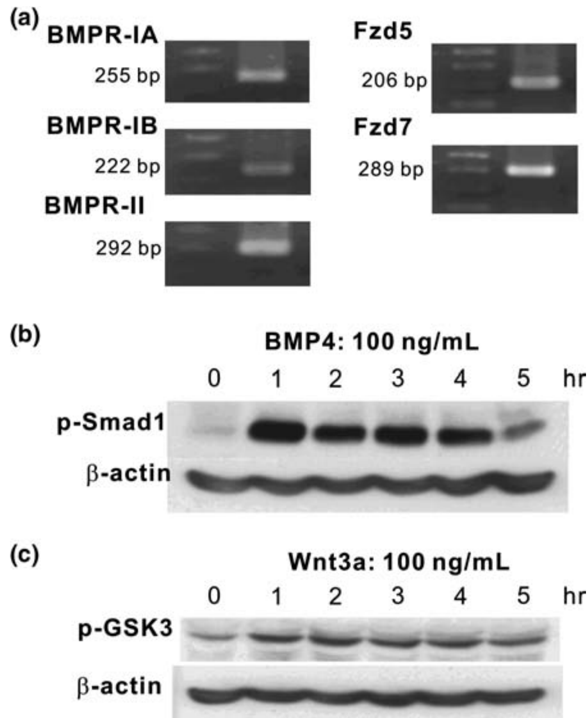

Figure 1. BMP and Wnt signalling in mouse embryonic stem cells (mESC). (a) mESCs were grown on 0.1% gelatin with normal mESC medium in the presence of 1000 units/mL LIF. Total RNA isolation of mESCs was performed when the cell growth was 80% confluent. Using RT-PCR to detect mRNA expression of BMP and Wnt receptors in mESCs, ethidium bromide-stained gels showed positive bands for BMP receptors (BMPR-IA, BMPR-IB and BMPR-II) and Wnt receptors (Fzd5 and Fzd7). (b) To avoid influence of LIF on the constitutive pathways in mESCs, cells grown on 0.1% gelatin were starved of LIF for 16 h before they were incubated with BMP4 100 ng/mL for the periods of time indicated. Immunoblotting showed that levels of phospho-Smad1 increased after 1 h of mESCs' incubation with BMP4. (c) Likewise, levels of phospho-GSK3 increased after 1 h, or more apparently 2 h, of mESCs' incubation with Wnt3a 100 ng/mL.

The ratio of Wnt3a to BMP4 doses is critical to their synergistic effects on proliferation of differentiating mESCs

Furthermore, to test whether the synergistic effect of BMP4 and Wnt3a on proliferation of differentiating mESCs depended on absolute doses of BMP4 and Wnt3a or their relative ratio of doses, four combinations of the dosages of the two factors were individually added to culture medium in the absence of LIF (Fig. 3a). Quantification of cell proliferation was performed by measurement of BrdU incorporation during DNA synthesis, based on colorimetric immunoassay. When Wnt3a 100 ng/mL was used, the synergistic effect between BMP4 and Wnt3A appeared to depend on doses of BMP4. Pro-proliferation effect on mESCs in the groups of W100B100 (Wnt3a 100 ng/mL + BMP4 100 ng/mL) and W100B150 (Wnt3a 100 ng/mL + BMP4 150 ng/mL) was much more pronounced than in groups W100B50 (Wnt3a 100 ng/mL + BMP4 50 ng/mL) and negative control (Fig. 3a). Although the W100B50 group seemed to have higher activity of cell proliferation compared to the control group, this difference did not reach statistical significance. Interestingly and unexpectedly, when Wnt3a dose was reduced to 50 ng/mL and at the same

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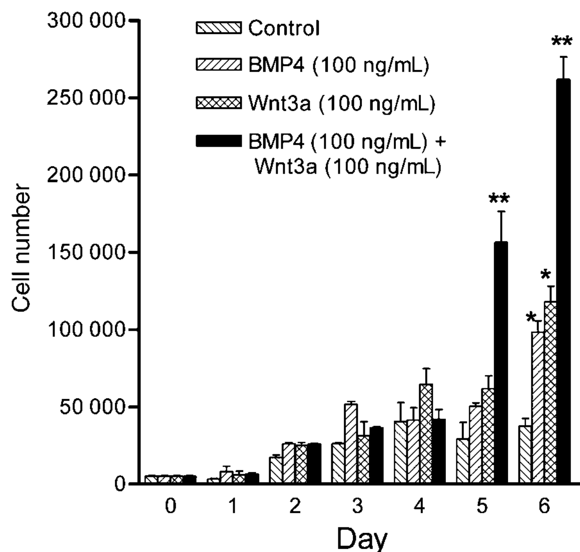

Figure 2. mESC proliferation in response to BMP4, Wnt3a or BMP4 + Wnt3a over 6 days. To define the effects of BMP4 and Wnt3a, and interaction between BMP4 and Wnt3a on mESC proliferation, mESCs cultured on 0.1% gelatin with mESC medium in the absence of LIF were separately treated with 100 ng/mL BMP4, 100 ng/mL Wnt3a, 100 ng/mL BMP4 + 100 ng/mL Wnt3a, or none of them (the control group). From days 0–4, there was no statistical difference in cell numbers of the four groups. On day 5, cell number of the group BMP4 + Wnt3a (156 453 ± 19 893) (mean ± SEM) was significantly greater than that of the control (29 408 ± 10711), $**P < 0.001$, and also those of the groups BMP4 (50 380 ± 2281) and Wnt3a (61 771 ± 8354), $*P < 0.01$. Furthermore, on day 6, although the groups BMP4 (98 361 ± 7439) and Wnt3a (118 093 ± 9884) had more cells when compared to the control (37 528 ± 5119), $*P < 0.01$, the cell number of the group BMP4 + Wn3a (261 960 ± 14 530) was markedly more than those of the other groups (the groups BMP4, Wnt3a and control), $**P < 0.001$. Shown are representative data of $n = 3$ experiments. Error bars represent SEM.

time, BMP4 50 ng/mL was used, that is, the group W50B50, the pro-proliferation effect rebounded to levels similar to those in W100B100 and W100B150, and much higher than those in W100B50 and the control (Fig. 3a). These data suggest that when the relative ratios of Wnt3a to BMP4 were ≤1 and also their individual doses were at least 50 ng/mL, their pro-proliferation effect on mESCs would be induced (Fig. 3i). Taken together, this indicates that relative ratio of Wnt3a to BMP4 doses is critical to their synergistic actions on proliferation of differentiating mESCs.

To confirm the individual contribution of BMP4 and Wnt3a to their synergistic actions on the proliferation of mESCs, we added the inhibitors of BMP4 and Wnt3a, that is noggin (250 ng/mL) and Dkk-1 (250 ng/mL), respectively, to the culture media. Noggin and Dkk-1 both were able to obliterate the proliferation-promoting effects of the combination of BMP4 and Wnt3a (Fig. 3a–c). These results further supported the existence of synergistic action between BMP4 and Wnt3a.

PI-3K, p38, ERK1/2 and JNK pathways are involved in synergistic effects between BMP4 and Wnt3a on proliferation of differentiating mESCs

Subsequently, we would like to define the mechanisms behind synergistic effects of BMP4 and Wnt3a on proliferation of differentiating mESCs. A recent study has reported that cross-talk

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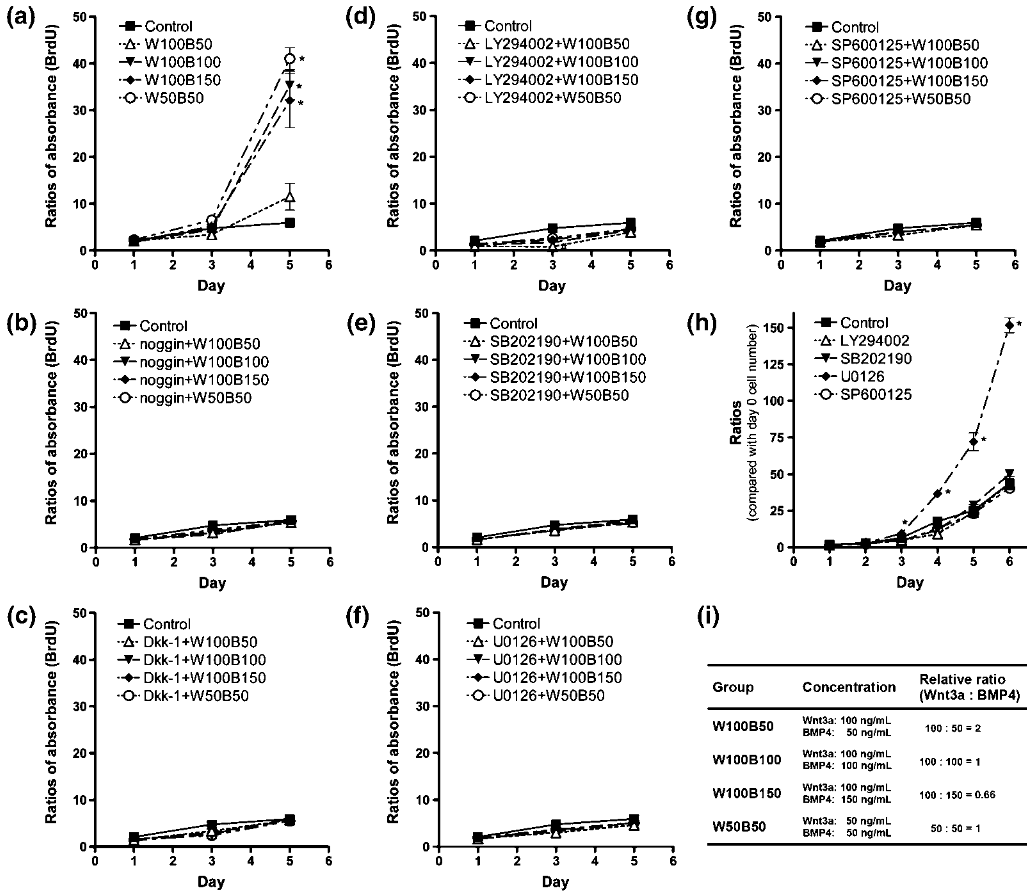

Group	Concentration	Relative ratio (Wnt3a : BMP4)
W100B50	Wnt3a: 100 ng/mL BMP4: 50 ng/mL	100 : 50 = 2
W100B100	Wnt3a: 100 ng/mL BMP4: 100 ng/mL	100 : 100 = 1
W100B150	Wnt3a: 100 ng/mL BMP4: 150 ng/mL	100 : 150 = 0.66
W50B50	Wnt3a: 50 ng/mL BMP4: 50 ng/mL	50 : 50 = 1

Figure 3. Synergistic effects of BMP4 and Wnt3a on mESC proliferation and the potential mechanisms behind these effects. (a–g) By using 5-bromo-2′-deoxyuridine (BrdU) incorporation assay, proliferation of mESCs was assessed in the following diverse treatments: control, with normal mESC medium in the absence of LIF and feeder cells; W100B50, adding Wnt3a 100 ng/mL + BMP4 50 ng/mL to the control medium; W100B100, adding Wnt3a 100 ng/mL + BMP4 100 ng/mL; W100B150, adding Wnt3a 100 ng/mL + BMP4 150 ng/mL; W50B50, adding Wnt3a 50 ng/mL + BMP4 50 ng/mL. In (b), noggin was added to each of the treatments excluding the control. In the same manner, noggin was used for (b), Dkk-1 (250 ng/mL), LY294002 (0.5 μM), SB202190 (2 μM), U0126 (10 μM) and SP600125 (1 μM) were used for (c–g), respectively. For (a–g), the Y-axis represents the absorbance (at 450 nm) ratio of the corresponding day to day 0, whereas the X-axis represents the day number. Absorbance in W100B100, W100B150 and W50B50 significantly increased when compared to W100B50 and control on day 5, $P < 0.05$ (a). This phenomenon was apparently abolished when differing inhibitors were added (b–g). (h) Genuine effect of LY294002 (0.5 μM), SB202190 (2 μM), U0126 (10 μM) and SP600125 (1 μM) was further explored by adding each single inhibitor to the control medium. Y-axis represents ratio of cell numbers of the corresponding day to day 0, and X-axis represents the day number. Interestingly, the cell number of the group U0126 was significantly greater than those of the other groups from days 3–6, $P < 0.01$. (i) The table elucidates the relative ratios of Wnt3a to BMP4 doses in W100B50, W100B100, W100B150 and W50B50.

between BMP and Wnt signal pathways in intestinal stem cells may be bridged through the PI-3K pathway (Tian *et al.* 2005). To determine whether the PI-3K pathway was involved in the combined effect of BMP4 and Wnt3a on enhancing proliferation of differentiating the cells, we cultured them with LY294002 (0.5 μM), the inhibitor of the PI-3K pathway, in the four groups,

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that is, W100B50, W100B100, W100B150 and W50B50 (Fig. 3d), and measured BrdU incorporation during DNA synthesis. Notably, LY294002 markedly suppressed enhanced proliferation effect on mESCs in the groups in which the concentration ratio of Wnt3a to BMP4 were ≤1 (Fig. 3a,d,i).

Given that p38, ERK1/2 and JNK pathways have been implicated in proliferation of stem cells (Lemonnier *et al*. 2004; Almeida *et al*. 2005; Jin *et al*. 2006), we further tested the involvement of these pathways in synergistic effects of BMP4 and Wnt3a on the proliferation of mESCs by adding the inhibitors of p38, ERK1/2 and JNK pathways, that is, SB202190 (2 μM), U0126 (10 μM) and SP600125 (1 μM), respectively, in groups W100B50, W100B100, W100B150 and W50B50. Cell proliferation was quantified by measurement of BrdU incorporation. Like LY294002, SB202190 (2 μM), U0126 (10 μM) and SP600125 (1 μM) efficiently abolished pro-proliferation actions of combinations of BMP4 and Wnt3a (Fig. 3a,e–g). Taken together, these results suggest that PI-3K, p38, ERK1/2 and JNK pathways may be involved in synergistic effects between BMP4 and Wnt3a on proliferation of differentiating mESCs.

Genuine effects of inhibitors of PI-3K, ERK1/2, p38 and JNK pathways on proliferation of differentiating mESCs

To further confirm the notion that synergistic effects between BMP4 and Wnt3a on proliferation of differentiating mESCs may work through PI-3K, p38, ERK1/2 and JNK pathways, we examined the genuine effects of inhibitors of these pathways on mESCs. In the absence of LIF, BMP4 and Wnt3a, we cultured mESCs with LY294002 (0.5 μM), SB202190 (2 μM), U0126 (10 μM) or SP600125 (1 μM) for 6 days. The effect on cell proliferation was then determined by cell counting (Fig. 3h). Compared to control (no inhibitor), LY294002, SB202190 and SP600125 did not enhance nor suppress mESC proliferation during the 6-day culture (Fig. 3h). Taken with the data that LY294002, SB202190 and SP600125 could repress enhanced proliferation of mESCs by combinations of BMP4 and Wnt3a, these results further supported that PI-3K, p38 and JNK pathways may exert most of their actions directly downstream in synergistic effect between BMP4 and Wnt3a. Notably, in contrast to inhibitors of PI-3K, p38 and JNK pathways, U0126 (10 μM) promoted proliferation of differentiating mESCs, compared to controls from day 3 to day 6 of culture (Fig. 3h). On the basis of this finding, we hypothesized that ERK1/2 pathway may play different roles in mESC proliferation, depending on whether there is synergistic effect of BMP4 and Wnt3a.

Integral effects of BMP4 and Wnt3a repressed differentiation of ectodermal lineage from mESCs whereas noggin, Dkk-1 and the inhibitors of PI-3K, ERK1/2, p38 and JNK pathways were able to reverse this effect

To define the integral effect of BMP4 and Wnt3a on differentiation of mESCs, we cultured them on gelatin for 6 days in the absence of LIF. The six kinds of inhibitors were added to the four basic conditions. This resulted in 29 separate conditions (24 modified conditions and the control) (Fig. 4). The four basic conditions were W100B50 (Wnt3a 100 ng/mL + BMP4 50 ng/mL), W100B100 (Wnt3a 100 ng/mL + BMP4100 ng/mL), W100B150 (Wnt3a 100 ng/mL + BMP4 150 ng/mL), and W50B50 (Wnt3a 50 ng/mL + BMP4 50 ng/mL). The six inhibitors used for testing integral effect of BMP4 and Wnt3a were noggin (250 ng/mL), Dkk-1 (250 ng/mL), LY294002 (0.5 μM), SB202190 (2 μM), U0126 (10 μM) and SP600125 (1 μM). After 6 days of culture, mESCs were harvested for total RNA extraction. Using RT-PCR to detect mRNA expression of Pax6 (ectoderm marker), Brachyury (mesoderm marker), HNF-4 (endoderm marker) and Stella (germ cell marker), we found that expression of Pax6 in the groups W100B50, W100B100, W100B150 and W50B50 was markedly lower when compared to the

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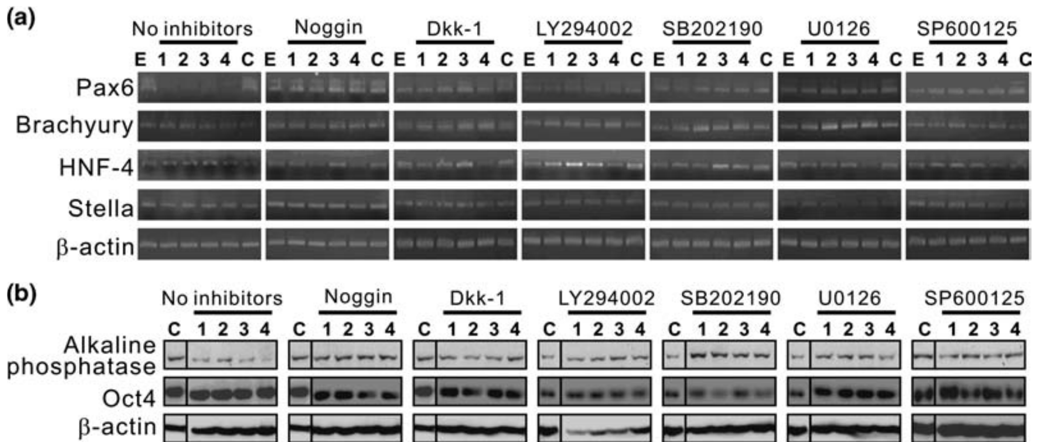

Figure 4. Effects of the varying combinations of BMP4 and Wnt3a on differentiation of mESCs after 6 days of culture. (a,b) Designations are explained as follows. C control group in which mESCs had been grown for 6 days on 0.1% gelatin with normal mESC medium but in the absence of LIF and feeder cells. E represents the group in which mESCs were maintained on 0.1% gelatin with normal mESC medium and LIF. 1, 2, 3 and 4 represent (Wnt3a 100 ng/mL + BMP4 50 ng/mL) (Wnt3a 100 ng/mL + BMP4 100 ng/mL) (Wnt3a 100 ng/mL + BMP4 150 ng/mL) and (Wnt3a 50 ng/mL + BMP4 50 ng/mL), respectively. The inhibitors were added in the following concentrations: noggin (250 ng/mL), Dkk-1 (250 ng/mL), LY294002 (0.5 μM), SB202190 (2 μM), U0126 (10 μM) and SP600125 (1 μM). (a) Using RT-PCR to detect the mRNA expression of Pax6, Brachyury, HNF-4 and Stella in mESCs after 6 days of culture, the ethidium bromide-stained gels showed that compared to the control, groups 1–4 had decreased expression of Pax6. Moreover, this reduction of Pax6 expression was reversed to that observed in the control when the various inhibitors were added. There seemed to be no difference for the expression of the other genes between the groups. (b) Western blots were performed to determine the expression of alkaline phosphatase and Oct-4 in mESCs. Compared to the control, the expression of alkaline phosphatase in groups 1–4 decreased markedly. In contrast, when the inhibitors were added, this expression increased to the level close to that in the control. Notably, the groups supplemented with LY294002, SB202190 and U0126 had higher expression levels than the control. Regarding the expression of Oct-4, there seemed to be no difference between the groups, except that the group supplemented with U0126 showed higher expression levels of Oct-4 when compared to the control.

control. Interestingly, when we separately added every single inhibitor to these four groups, Pax6 expression for each group was elevated to the level similar to that in the control (Fig. 4a). In contrast to Pax6 expression, mRNA expression of Brachyury, HNF-4 or Stella for each culture condition did not vary greatly from the control (Fig. 4a). Taken together, these results indicate that integral action of BMP4 and Wnt3a may restrain differentiation of ectodermal lineage development from mESCs.

Integral effect of BMP4 and Wnt3a on expression of alkaline phosphatase and Oct-4

We also examined whether there was difference in expression of self-renewal genes (e.g. alkaline phosphatase and Oct-4), for each culture condition by Western blotting. Compared to controls, the combination of BMP4 and Wnt3a, including W100B50, W100B100, W100B150 and W50B50, reduced expression of alkaline phosphatase (Fig. 4b). When inhibitors were added to the combinations of BMP4 and Wnt3a, we found that all of them were able to reverse expression levels of alkaline phosphatase up to at least that of the control. Specifically, LY294002, SB202190 and U0126 not only negated the integral effect of BMP4 and Wnt3a on expression of alkaline phosphatase, but augmented it to a level more than that of the control (Fig. 4b). Notably,

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there seemed to be no detectable difference in Oct-4 expression between the different culture conditions and the control, except that the Oct-4 expression increased when U0126 was added to the combinations of BMP4 and Wnt3a (Fig. 4b).

DISCUSSION

Our results clearly demonstrate that BMP4 synergizes with Wnt3a to enhance proliferation of differentiating mESCs. This has further been validated by the inhibitor experiments, showing that noggin or Dkk1 was able to efficiently abolish such synergistic effects. These findings are consistent with recent reports indicating potential synergism between BMP and Wnt signalling, for example, they have been shown to act in concert to regulate Emx2 transcription in governing pattern formation of dorsal telencephalon in mouse embryological development (Theil *et al.* 2002). Similarly, cooperation between BMP and Wnt signalling plays a key role in control of formation of apical ectodermal ridge and dorsal–ventral axis in the limb (Soshnikova *et al.* 2003). Nishanian *et al.* explored the actions of BMP4 on suppression of tumourigenesis using human cancer cells and proposed the importance of the roles of the interaction between BMP and Wnt signalling in tumour suppression (Nishanian *et al.* 2004).

Notably, we further demonstrated that the relative ratio of Wnt3a to BMP4 doses was crucial for their synergy of effect on proliferation of differentiating mESCs. This result can be explained by the concept of morphogens whose local concentrations will determine their effects. It is well recognized that BMP and Wnt both play roles as morphogens during development (Christian 2000). Moreover, considering that Wnt and BMP influence many developmental processes, and that some downstream effects of Wnt and BMP signalling may act synergistically (Theil *et al.* 2002; Mbalaviele *et al.* 2005) whereas others may act antagonistically (He *et al.* 2004), relative ratios of Wnt to BMP concentration may be pivotal to determine integral effects from Wnt and BMP signalling. Our data point to the importance of the relative ratio of Wnt3a to BMP4 doses, especially its role on proliferation of differentiating mESCs. The concept arising from our findings could be applied to the technology for *in vitro* expansion of ESCs. However, before the application can be consolidated, changes in receptor abundance and individual downstream signalling effects induced by Wnt3a and BMP4 should be further investigated.

In an attempt to define mechanisms behind the synergism of Wnt3a and BMP4, we found that inhibitors for PI-3K, p38, ERK1/2 and JNK pathways were able to abolish synergistic effects from Wnt3a and BMP4 on proliferation of differentiating mESCs, indicating involvement of these pathways in cooperation of Wnt3a and BMP4. These data were in keeping with recent observations that the PI-3K pathway appeared to function as a bridge of cross-talk between BMP and Wnt signalling in intestine stem cells (Tian *et al.* 2005). Moreover, Jin *et al.* (2000) have reported that BMP signalling works through p38 pathway to modulate Wnt signal transduction during chondrogenesis in chick wing bud mesenchymal cells. ERK1/2 pathway has been implicated in BMP induced osteoblast differentiation of mesenchymal C3H10T1/2 progenitor cells (Lou *et al.* 2000). In addition, Yun *et al.* (2005) have demonstrated that Wnt3a was able to enhance cell proliferation in NIH3T3 fibroblast cells *via* ERK1/2 pathway. In addition, JNK pathway has been involved in BMP induced osteoblastic cell differentiation (Lemonnier *et al.* 2004) and Wnt expression in neural differentiation (Amura *et al.* 2005). Collectively, these recent studies suggested that PI-3K, p38, ERK1/2 and JNK pathways were associated with BMP and Wnt signalling in various types of cells, thereby indirectly supporting our data that were focused on the differentiating mESCs. However, given diversity of the function of BMP and Wnt

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signalling, it is clear that further studies are necessary to explore other potential mechanisms working behind the synergy of BMP and Wnt signalling in mESCs, as well as to delineate the details of influence from PI-3K, p38, ERK1/2 and JNK pathways. It would also be useful, in an *in vivo* system, to decipher what factors might modulate both the bioavailability of Wnt and BMP signalling and their relative levels.

Our data from the use of RT-PCR for differentiating mESCs suggested that the integral effect of Wnt3a and BMP4 repressed ectodermal differentiation of mESCs, since the mRNA expression of Pax6 (ectoderm marker) significantly decreased whereas those of Brachyury (mesoderm marker), HNF-4 (endoderm marker) and Stella (germ cell marker) did not change obviously after 6 days culture with Wnt3a and BMP4, when compared to the control. In contrast to the synergistic effect of Wnt3a and BMP4 on cell proliferation, the relative ratio of Wnt3a to BMP4 doses did not affect the combination effect of Wnt3a and BMP4 on expression profiles of differentiation of mESCs. It is of note that the inhibitors for PI-3K, p38, ERK1/2 and JNK pathways were able to reverse the expression levels of Pax6 to that of the control, suggesting that these pathways may be involved in inhibition of ectodermal differentiation of mESCs induced by combination of Wnt3a and BMP4. The point that integral, not individual, effects of Wnt3a and BMP4 suppressed ectodermal differentiation of mESCs was further confirmed in the experiments using individual inhibitors of Wnt3a and BMP4, Dkk-1 and noggin, respectively, was able to abolish the suppression effect from combination of Wnt3a and BMP4. It would be interesting to further dissect what types of differentiated cells would be repressed from differentiation of mESCs by the combination influence of Wnt3a and BMP4 in our further studies.

In summary, our results provide further insight into the potential cooperative actions of Wnt3a and BMP4 on differentiating mESCs. We clearly demonstrate that Wnt3a synergized with BMP4 to enhance proliferation of differentiating mESCs. Furthermore, the relative ratio of Wnt3a to BMP4 doses is critical to the synergistic effect, which may work through PI-3K, p38, ERK1/2 and JNK pathways. It is of note that the combination effect of Wnt3a and BMP4 could restrain ectodermal differentiation from mESCs. This effect could be reversed by use of the inhibitors of PI-3K, p38, ERK1/2 or JNK pathways. Taken together, these data incite interest to further decipher the actions of Wnt and BMP signalling on ESCs. It might also help construct a new concept concerning the combination effect of Wnt3a and BMP4 on the differentiating mESCs.

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