論文内容の要旨

論文題目 Role of p38 Mitogen-Activated Protein Kinase in lineage commitment of mouse ES cells
(p38 MAP キナーゼのマウス ES 細胞分化決定における役割)

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[INTRODUCTION]

Embryonic Stem (ES) cells are able to differentiate into a variety of cell types, which provide an attractive source of cells for disease modeling in vitro and the treatment of many degenerative diseases. An essential step for therapeutic and research applications of stem cells is the ability to differentiate them into specific cell types. Understanding the mechanisms of ES cell differentiation is crucial for developing more efficient and selective methods to direct the differentiation of ES cells to produce homogenous populations of particular cell types. However, molecular mechanisms that control the commitment of ES cells into a specific lineage are still poorly understood. In this study, we will demonstrate the physiological roles of p38 mitogen-activated protein kinase (p38 MAPK) in lineage commitment of mouse ES cells.

[METHODS AND RESULTS]

p38 MAPK specific inhibitor SB203580 blocked cardiomyogenesis and committed ES cells into the neuronal lineage

Mouse ES cells are maintained in an undifferentiated state and retain the potential for unlimited proliferation in the presence of Leukemiainhibitory Factor (LIF). Removing LIF and cultured in serum containing medium, ES cells can be differentiated spontaneously into beating cardiomyocytes through embryonic body (EB) formation to mimic embryo development in vivo. Mitogen-activated protein (MAP) kinases are serine/threonine-specific

protein kinases that respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis. To investigate the role of three main MAP kinase and PI3- kinases in ES cell lineage commitment, EBs were treated with specific inhibitors between day 1 and day 6 during the differentiation process. The effect of the extracellular signal-regulated kinase (ERK)-specific inhibitor U0126, stress-activated protein kinase/c-Jun NH2-terminal kinase (JNK)-specific inhibitor SP6000125, p38 MAPK-specific inhibitor SB203580, and Phosphoinositide 3-kinase (PI3K)-specific inhibitor wortmannin, were evaluated. We found that ERK-specific inhibitor U0126 and p38 MAPK-specific inhibitor SB203580 significantly blocked spontaneous cardiac differentiation, with less than 5% of EBs containing beating foci at day 12. In contrast, more than 90% of untreated control EBs containing beating areas, which were confirmed with phenotypic observations (Fig. 1A). Interestingly, more than 90% of SB203580 treated EBs exhibited prominent growth when blocked cardiomyogenesis (Fig. 1B). These outgrowths showed positive immunofluorescence staining with anti-βIII-tubulin antibody specific for neurons, whereas EBs treated with U0126 did not, which has the same effect on the inhibition of cardiac differentiation (Fig. 1C). These results demonstrate that the neural differentiation is induced specifically by SB203580, but does not due to the inhibition of cardiomyogenesis.

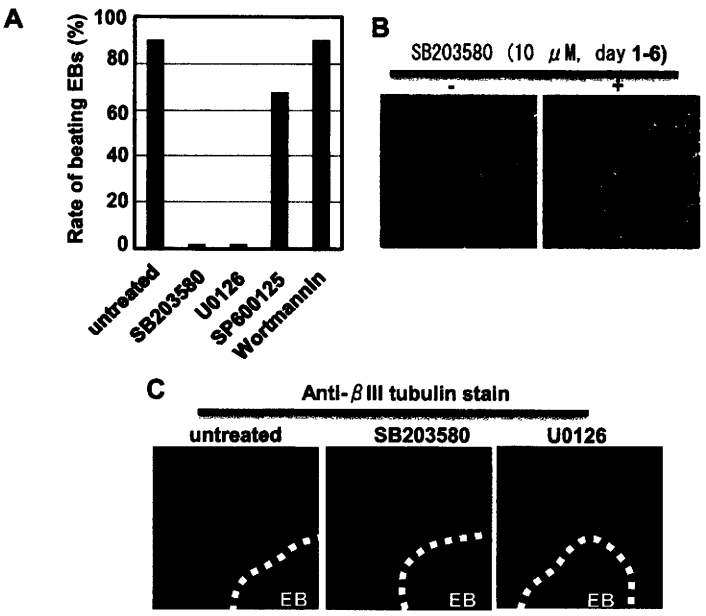

Fig. 1 . Effects of MAP kinase specific inhibitors on ES cell differentiation.

To further confirm the switch effect of SB203580 on cardiac versus neural differentiation, frozen sections from EBs treated with or without SB203580 between day 1 and day 6 were immunohistochemical staining at day 12. EBs with SB203580 treatment did not stain with a cardiac-specific marker α actinin, but did stain positively with anti-α TUJ antibody specific for neurons. And RT-PCR analysis showed that SB203580 treatment completely inhibited the expression of cardiac genes, including *mef2c*, *mhc*, and *mlc2v*, whereas induced significant increase in mRNA level of neural-specific genes, *nestin*, *hes5*, *mash1*, *mash3*, and *map2*.

p38 MAPK activity between day 3 and 4 determines cardiac or neural commitment of ES cells

To investigate the role of p38 MAPK in ES cell commitment, we first measured the p38 MAPK activity during

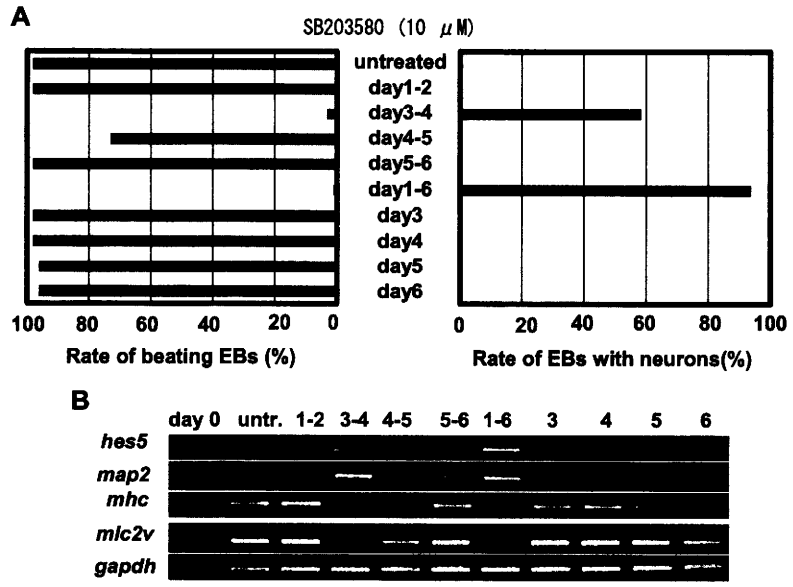

Fig. 2 SB203580 treatment between day 3 and 4 specifically blocks cardiomyogenesis and induces neurogenesis.

early stages of differentiation by Western blot using anti-phospho-p38 MAPK and anti-p38 MAPK antibodies. At day 0, when ES cells were cultured as monolayer, no detectable p38 MAPK activation was observed in whole cell lysates. Interestingly, after the EB formation, p38 MAPK was spontaneously activated and peaked from day 2 to day 6 without affecting the expression level of p38 MAPK protein. Next we determined at what time point p38 MAPK acts during the differentiation process by treating EBs with SB203580 at various time intervals. The treatment of SB203580 between day 3 and day 4 significantly promoted neuron differentiation, as did a treatment from day 1 to day 6, whereas SB203580 treated at other periods did not induce neurogenesis (Fig. 2A). RT-PCR analysis confirmed that the expression of neuronal markers *hes5* and *map2* were induced only when the inhibitor was applied between day 3 and day 4 or between day 1 and day 6, while the cardiomyocyte-specific genes *mhc* and *mlc2v* were strongly decreased (Fig. 2B). These results clearly demonstrated that the p38 MAPK activity between day 3 and 4 is critical for cardiac or neural commitment of ES cells.

p38 MAPK functions as a molecular switch in ES cell commitment into cardiomyocytes versus neurons by controlling the expression of BMP-2

To further elucidate the molecular mechanism of p38 MAPK in governing the cell-fate choices in ES cells, we sought to identify downstream targets of p38 MAPK. Since p38 MAPK activity between day 3 and 4 is crucial for the lineage commitment, we investigated whether the mRNA expression of known genes involved in cell fate determination were affected by treating the EBs with or without SB203580 between day 3 and 4. Notably, RT-PCR analysis showed that the mRNA expression of bone morphogenetic protein 2 (*bmp-2*) was decreased with the SB203580 treatment. Further analysis revealed that *bmp-2* was induced from day 4 and SB203580 treatment strongly repressed this induction.

To determine whether downregulation of BMP-2 expression was a key factor for SB203580 to induce

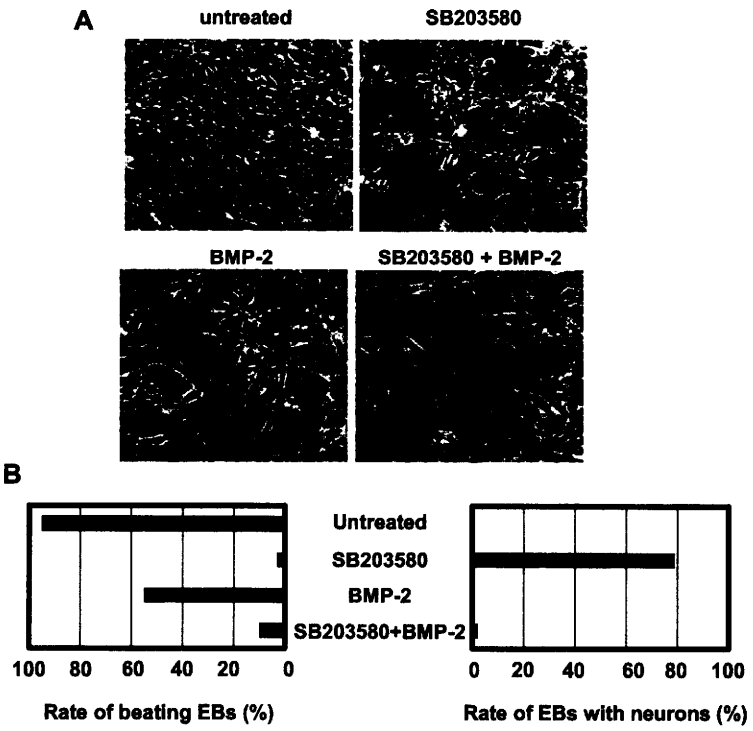

Fig. 3 BMP-2 inhibits SB203580-induced neurogenesis.

neurogenesis, recombinant human bone morphogenetic protein-2 (rhBMP-2) was applied to the EBs from day 4 to 6 in the presence of SB203580 from day 3 to 6. Predictably, rhBMP-2 remarkably repressed the neuron differentiation induced by SB203580 treatment (Fig. 3A). Quantification analysis indicated that SB203580 treatment from day 3 to 6 resulted in nearly 80% of neural induction, whereas addition of rhBMP-2 decreased this rate to less than 5% (Fig. 3B). Consistent with the microscopic analysis, RT-PCR confirmed that rhBMP-2 strongly inhibited the expression of neuron-specific gene map2 induced by SB203580 Meanwhile, the expression of cardiomyocyte-specific genes *mhc* and *mlc2v* were slightly recovered by the addition of rhBMP-2. Furthermore, treated the EBs with BMP-2 antagonist Noggin blocked the cardiomyogenesis, conversely, promoted neuronal differentiation, similar to the SB203580 treatment. These results indicate that p38 MAPK controls ES cell lineage commitment by regulating the expression of BMP-2.

BMP-2 is a direct transcriptional target of MEF2C, a well-known substrate of p38 MAPK

SB203580 treatment also resulted in a dramatic decrease in *mef2c* expression, which had a similar expression pattern as *bmp-2* during the differentiation process. MEF2C is a crucial transcription factor controlling the activation of many cardiac-specific genes. Interestingly, p38 MAPK is a well-known regulator of MEF2C, which can phosphorylate MEF2C directly on residues located within the activation domain, suggesting that p38 MAPK may induce cardiomyogenesis via direct regulation of MEF2C. Moreover, highly conserved consensus-binding site for MEF2 was detected in both mouse and human proximal *bmp-2* promoters. Therefore, we speculate that MEF2C may directly regulate the transcription of *bmp-2*. To test this hypothesis, we first carried out luciferase reporter assays with HeLa cells using a proximal promoter region (−1703/−1 bp) of mouse *bmp-2* containing the MEF2 site. Overexpression of MEF2C increased luciferase activity 3-fold, whereas SB203580

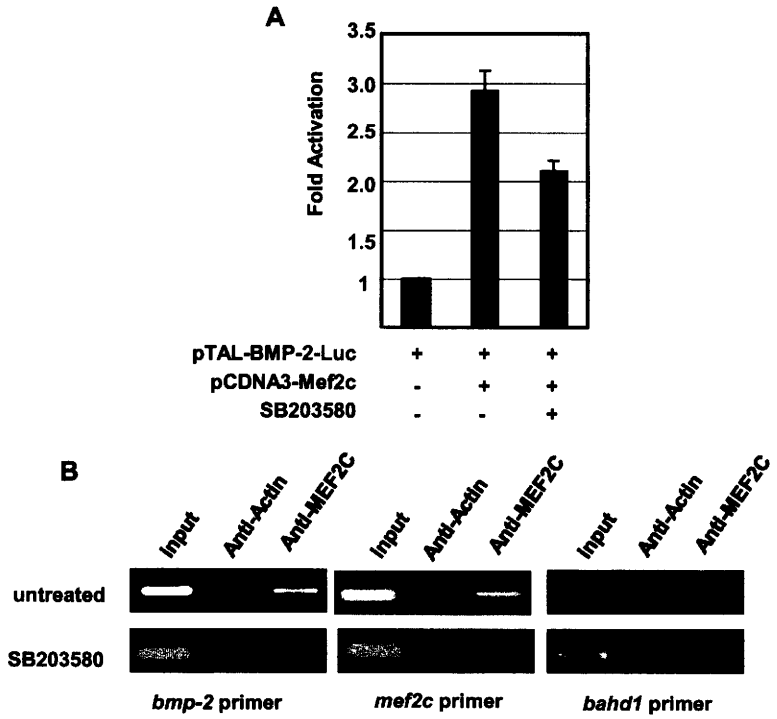

Fig. 4 MEF2C controls BMP-2 promoter activity.

treatment repressed its transactivation (Fig. 4A). To determine whether MEF2C can bind to the *bmp-2* promoter region, chromatin immunoprecipitation (ChIP) analysis was performed at day 6 under spontaneous differentiation conditions. The mouse *bmp-2* promoter region encompassing the −656/−635 bp MEF2 site was precipitated in the presence of an anti-MEF2C specific antibody, indicating that MEF2C can bind to the *bmp-2* promoter(Fig. 4B, up-left). but does not bind to the promoter region of control *bahd1* gene present on the same chromosome (Fig. 4B, up-right). Furthermore, SB203580 treatment inhibited this binding of MEF2C to the MEF2 site (Fig. 4B, down-left). As a positive control, the region promoter mouse mef2c, which itself is a target of MEF2 transcription factor, was also precipitated similar to *bmp-2* (Fig. 4B, middle). These data suggest that *bmp-2* is a potential transcriptional target of MEF2C.

[CONCLUSIONS]

In this study, our results demonstrate that p38 MAPK functions as a molecular switch in ES cell commitment into cardiomyocytes versus neurons. During the differentiation process, the high p38 MAPK activity between day 3 and 4 is crucial for cardiomyogenesis of ES cells, and specific inhibition of this activity can direct the differentiation of ES cells from cardiomyocytes toward neurons. Furthermore, our data show that BMP-2 is a critical downstream target of p38 MAPK and appears to be regulated directly by transcription factor MEF2C, a well-known substrate of p38 MAPK.