Biochemical and Biophysical Research Communications 401 (2010) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibitory Smad proteins promote the differentiation of mouse embryonic stem cells into ependymal-like ciliated cells

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ARTICLE INFO

Article history:
Received 24 August 2010
Available online 31 August 2010

Keywords:
Ciliated cells
Embryonic stem cells
Bone morphogenetic protein
Smad7
Smad6
Musashi1

ABSTRACT

Motile cilia play crucial roles in the maintenance of homeostasis *in vivo*. Defects in the biosynthesis of cilia cause immotile cilia syndrome, also known as primary ciliary dyskinesia (PCD), which is associated with a variety of complex diseases. In this study, we found that inhibitory Smad proteins, Smad7 and Smad6, significantly promoted the differentiation of mouse embryonic stem (ES) cells into ciliated cells. Moreover, these Smad proteins specifically induced morphologically distinct Musashi1-positive ciliated cells. These results suggest that inhibitory Smad proteins could be important regulators not only for the regulation of ciliated cell differentiation, but also for the subtype specification of ciliated cells during differentiation from mouse ES cells.

1. Introduction

Mammalian cilia are structurally classified as 9 + 0 or 9 + 2 on the basis of microtubule arrangement. Primary cilia (9 + 0) generally occur singly per cell and are immotile due to a lack of axonemal dyneins. Primary cilia exist in almost all cells. In contrast, 9 + 2 cilia normally occur in multiples and have axonemal dyneins, which provides motility. These 9 + 2 cilia are observed in specific regions of the body such as the respiratory tract, reproductive organs, and brain ependyma [1]. Defects in the structural components of cilia have been reported to cause immotile cilia syndrome, also known as primary ciliary dyskinesia (PCD), which is associated with various diseases such as polycystic kidney disease, chronic bronchitis, sinusitis, situs inversus, male sterility, and polydactyly [2]. Moreover, cilia play crucial roles in the differentiation and survival of olfactory and retinal neurons and auditory hair cells [3]. Cilia are also involved in cell–cell communication, including Shh signaling, non-canonical and canonical Wnt signaling, and platelet derived growth factor (PDGF)-mediated signaling events [4].

The biogenesis of cilia has been morphologically characterized by a number of model systems. The essential genes involved in the biogenesis of basal bodies and cilia have been identified by genetic studies with *Chlamydomonas* and *Caenorhabditis elegans*, bioinformatic and proteomic studies, and studies on human disease [2,4,5]. However, the knowledge of ciliogenesis is still largely limited to ultrastructural observations and scattered information on various genes. Moreover, the regulatory signaling pathways involved in the development of ciliated cells have not been elucidated in detail. During early lung development, Foxj1 was shown to be a critical transcription factor on basis of the lack of ciliated epithelium in Foxj1-knockout mice [6]. The importance of Foxj1 was further confirmed with transgenic mice expressing Foxj1 in the distal endoderm of developing lung, which showed ectopic development of ciliated epithelium in distal airways [7]. Recently, Notch signaling has been reported to be another important regulator for establishing bronchiolar epithelial cell lineages. Inactivation of Notch signaling early during lung development led to expansion of ciliated and neuroendocrine lineages [8]. In contrast, an activated Notch1 intracellular domain in the lung epithelium decreased ciliated cell differentiation [9].

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doi:10.1016/j.bbrc.2010.08.099

In this study, taking advantage of a recently developed method for *in vitro* differentiation of mouse embryonic stem (ES) cells [10], we found that ectopic expression of Smad7 and Smad6, inhibitors of bone morphogenetic protein (BMP) signaling, dramatically enhanced the *in vitro* differentiation of ES cells into ciliated cells. Moreover, the induced ciliated cells had characteristic long cilia on the cell surface and the cells were positive for Musashi1, a neural stem cell marker. Our results suggest that inhibitory Smad proteins control the early differentiation of ciliated cells and promote their differentiation into a specific ciliated cell type *in vitro*.

2. Materials and methods

2.1. Establishment of tetracycline-regulated Smad7- and Smad6-expressing ES cell lines

Tetracycline (Tc)-regulated Smad6- and Smad7-expressing ES cell lines were established according to the method of Masui et al. [11]. The procedure involved 2 steps: the knock-in step to insert the ROSA-TET locus and an exchange step with the Tc-regulatable expression vector. To create the ROSA-TET locus, mouse ES cells (E14) were electroporated with linearized pMWROSATcH DNA. Hygromycin-resistant ES clones that contain the ROSA-TET locus were selected in a medium containing 200 μg/ml hygromycin (Invitrogen). The open reading frames of Smad7 and Smad6 were obtained from pcDNA3-Flag-mouse Smad7 and Smad6, respectively, by digestion with *BamHI* and *XhoI*, blunting with T4 polymerase, and subcloning into the *EcoRV* site of pBluescript-SK (−). The *XhoI–NotI* fragments of Flag-Smad7 or Flag-Smad6 DNA were then subcloned into the exchange vector pPthC. The knock-in ES cells were transfected with pPthC-Flag-Smad7 or pPthC-Flag-Smad6 with pCAGGS-Cre by using Lipofectamine 2000 (Invitrogen) and cultured in the presence of 1.5 μg/ml puromycin (Sigma) and 1 μg/ml Tc. The established ES clones were cultured in ES medium (see below) with or without Tc, and the expression of exogenous genes was confirmed by coexpression of the fluorescent protein, Venus, using epifluorescence microscopy. The Tc-regulated Smad7- and Smad6-expressing ES cell lines were maintained on a feeder cell layer of puromycin-resistant, mitomycin C-inactivated mouse embryonic fibroblasts (the DR4 line from the ATCC). ES cells were maintained in ES medium (DMEM (high glucose) containing 15% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 0.1 mM beta-mercaptoethanol, 100 U/ml penicillin, 100 U/ml streptomycin, and 1000 U/ml leukemia inhibitory factor (Chemicon)) supplemented with 1.5 μg/ml puromycin. The medium was changed daily, and the cells were passaged every 3 days.

2.2. Differentiation of ES cells

Trypsinized and dissociated ES cells were incubated for 30 min on a culture dish to remove the feeder cells. They were then seeded onto round-bottomed low-attachment 96-well plates (Nunc) at 2000 cells/well in 10% FBS-DMEM containing 1.5 μg/ml puromycin and 1 μg/ml Tc and incubated for 3 days to form ES cell aggregates (embryoid bodies, EBs). The EBs were transferred to gelatin-coated dishes (10 EBs/60-mm dish) for attachment and cultured in 10% FBS- or 10% knockout serum replacement (KSR)-containing DMEM supplemented with 1.5 μg/ml puromycin with or without 1 μg/ml Tc. When testing the effect of Smad7 and Smad6 proteins on the differentiation of ciliated cells, these inhibitory Smad proteins were expressed for the first 6 days of attachment culture because the inhibitory effects of FBS on the differentiation of ciliated cells are only observed in the early stages of attachment culturing [10]. To test the neutralizing effect of BMP4 antibody on the differentiation of ES cells, anti-BMP4 antibody (R&D) was used.

2.3. Immunocytochemical and flow cytometric analyses

Immunocytochemical analysis of EBs was performed as described previously [10]. The following antibodies were used in this study: mouse monoclonal antibody against beta-tubulin IV (BioGenex), rat monoclonal antibody for Musashi1 (kind gift from Dr. H. Okano, Keio University), and rabbit polyclonal antibody for S100 (DAKO).

For flow cytometric analysis, EBs cultured for 25–30 days were washed twice with low-calcium buffer [10 mM Hepes (pH 6.9), 120 mM NaCl, 5.4 mM KCl, 5 mM $MgSO_4$, 5 mM Na-pyruvate, 20 mM glucose, and 20 mM taurine] and then incubated in 1 ml of 0.9 mg/ml Blendzyme (Roche) at 37 °C for 30 min, which was added to the same buffer supplemented with 30 μM $CaCl_2$. After addition of the resuspending solution (85 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 1 mM EGTA, 2 mM ATP, 5 mM Na-pyruvate, 5 mM creatine, 220 mM taurine, and 20 mM glucose (pH 7.2)) to the medium, the EBs were incubated at 37 °C for 15 min and then further dissociated by pipetting. The dissociated cells were fixed with 4% paraformaldehyde in PBS for 20 min. After washing with PBS, the cells were permeabilized with PBS containing 0.5% Triton X-100 for 5 min and incubated in 3% BSA in PBS for 20 min. The cells were then immunostained with anti-beta-tubulin IV antibody as described in the immunocytochemical analysis. The expression of beta-tubulin IV was analyzed using an EPICS ALTRA (Beckman Coulter).

2.4. Western blotting

Preparation of the cell lysate and Western blotting analyses were performed as described previously [12]. The following antibodies were used in this study: anti-Flag antibody (1:1000; Affinity BioReagents), anti-alpha-tubulin antibody (1:10,000; Sigma), anti-Smad1/2/3 antibody (1:1000; Santa Cruz), anti-phosphorylated Smad1/5/8 antibody (1:1000; Cell Signaling), and anti-phosphorylated Smad2 antibody (1:250; Zymed Laboratories).

2.5. qRT-PCR

Total RNA was prepared as described previously [10], and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed as previously described [13]. The sequences of the primers used were as follows: *Foxj1*: forward 5′-gtttcatccacccatgttcc-3′, reverse 5′-gtaggacccttctgggcttc-3′, *beta-tubulin IV*: forward 5′-tcactctggctcccgtaagt-3′, reverse 5′-aggcaacaggcctctcttttt-3′, *centrin4*: forward 5′-aactgtgagccccaaaacag-3′, reverse 5′-gatctgggggccaagtttat-3′.

2.6. Electron microscopy

EBs were fixed with 0.1 M sodium cacodylate buffer (pH 7.4) containing 4% paraformaldehyde for 30 min and washed with 0.2 M sodium cacodylate buffer three times. EBs were postfixed with 1% osmium tetroxide for 30 min, dehydrated through an ethanol and acetone series, and then embedded in epoxy resin. Ultrathin sections cut at 80–90 nm thickness were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (JEM-200CX; JEOL, Tokyo).

3. Results

We have previously reported an *in vitro* method for inducing ciliated cell differentiation, whereby mouse ES cell aggregates were cultured on gelatin-coated dishes in serum-free medium [10]. We have further explored differentiation methodologies for cili-

ated cells and found that at least 2 types of ciliated cells were induced from mouse ES cells. Although both had multiple cilia, the major type (type I; 50–70% of the total ciliated cells) had longer cilia that were limited in number (approximately 5–30 cilia per cell) and the cilia were projected from a localized area of the cell surface (Fig. 1A and B, left). In contrast, the relatively minor type of ciliated cells (type II) had many short cilia (around 30–100 cilia per cell) that covered the full apical surface of the cell (Fig. 1A and B, right). Both type I and type II cells had beta-tubulin-IV-positive cilia, and they consistently expressed S100, a calcium-binding protein (Fig. 1C), which is expressed in various ciliated cells including trachea [14] and ependymal cells [15]. Interestingly, only type I cells expressed a neural stem cell marker, Musashi1 (Fig. 1D), which is also expressed in developing ependymal cells [16,17].

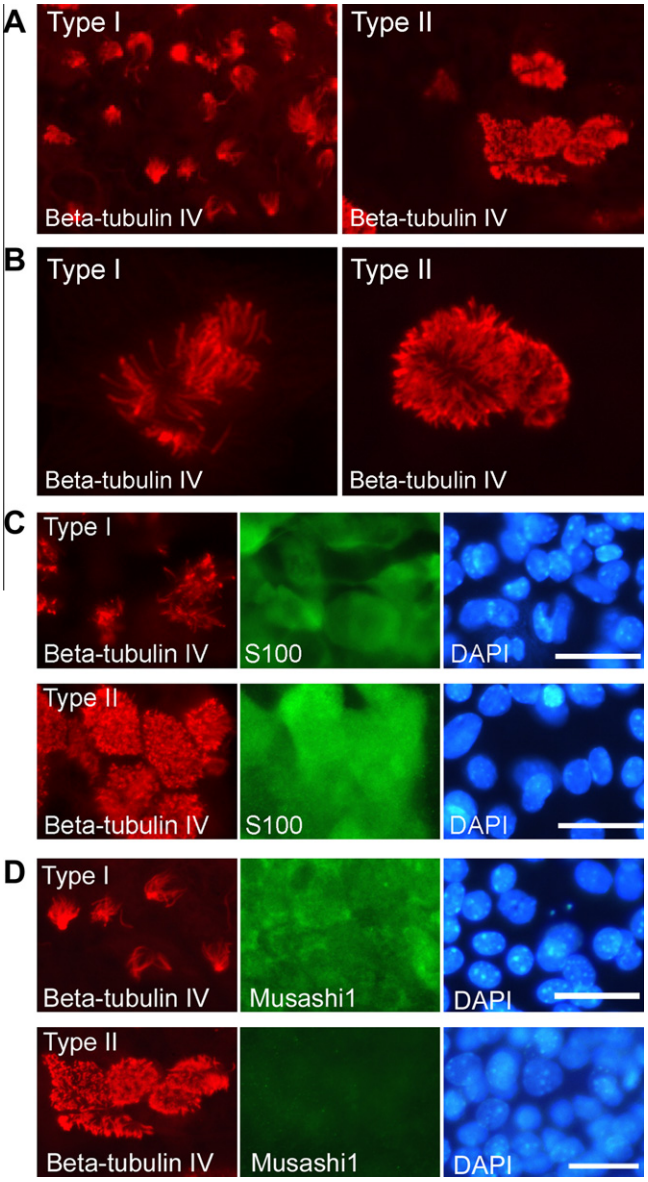

Fig. 1. Differentiation of mouse ES cells into 2 types of ciliated cells. Immunocytochemical analysis was performed on the ciliated cells differentiated in KSR medium for 25 days. The cells were immunostained with beta-tubulin IV (red) and S100 (green) or Musashi1 (green) antibodies. (A,B, left) Type I ciliated cells with characteristic elongated cilia. (A,B, right) Type II ciliated cells with numerous short cilia. Figures in (B) are the high magnification images. (C) Both type I and type II ciliated cells were positive for S100. (D) Type I ciliated cells were positive for Musashi1, but type II ciliated cells were negative for Musashi1. Scale bars; 25 μm.

Flow cytometric analysis revealed that approximately 10% of the cells were differentiated into beta-tubulin-IV-positive mature

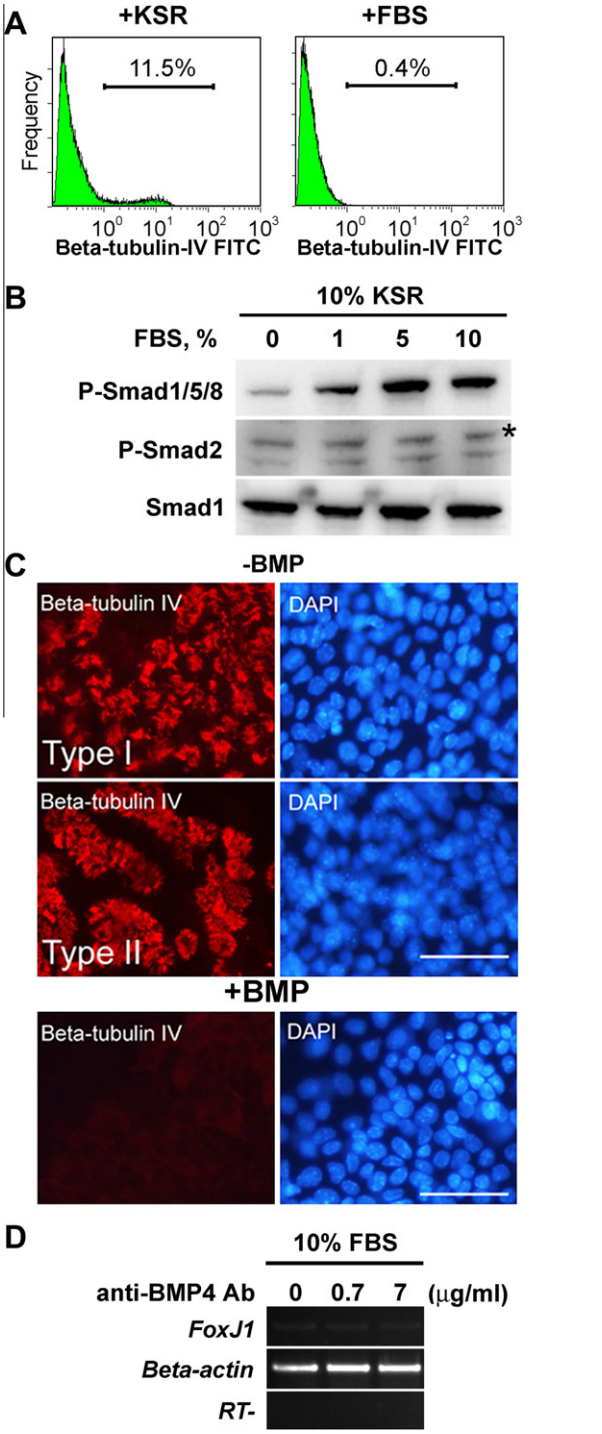

Fig. 2. Exhibition of BMP-like activities by FBS. (A) Flow cytometric analysis for the expression of beta-tubulin IV in differentiated cells cultured in KSR- or FBS-containing medium for 25 days. Beta-tubulin IV-positive cells were strongly inhibited in the FBS-containing medium. (B) ES cells were cultured in KSR–DMEM medium supplemented with increasing concentrations of FBS for 6 days. Phosphorylation of Smad proteins was analyzed by Western blotting. Smad1/5/8 was phosphorylated on addition of FBS in a dose-dependent manner. The phosphorylation of Smad2 was not altered by the addition of FBS. The asterisk indicates a nonspecific band. (C) Immunocytochemical analysis of differentiated ciliated cells in the control KSR-containing medium or in the KSR medium supplemented with BMP4. Scale bars; 50 μm. (D) *Foxj1* expression of EBs cultured in FBS medium supplemented with anti-BMP4 antibody. Addition of anti-BMP4 antibody did not increase *Foxj1* mRNA expression in the presence of 10% FBS.

ciliated cells, including type I and type II cells, by this differentiation method (Fig. 2A, left). In contrast, the medium with FBS strongly inhibited the differentiation of ciliated cells, reducing the efficiency to 0.4% (Fig. 2A right). Since FBS has been suggested to contain a small amount of BMP-like activity [18], we reasoned that BMP-induced signals in FBS might inhibit the differentiation of ciliated cells. In fact, EBs cultured in the serum-free medium supplemented with increasing concentrations (1–10%) of FBS showed a dose-dependent increase in the phosphorylation of a BMP-specific intracellular signaling molecule, Smad1/5/8 (Fig. 2B). In contrast, Smad2, which is specifically phosphorylated in the activin/TGFβ signaling pathway, showed only basal levels of phosphorylation even at high doses of FBS. When BMP4 was added in a serum-free culture medium during differentiation, formation of both type I and type II cells was equally inhibited (Fig. 2C). These results suggest that culturing EBs in FBS-containing medium elicits significant BMP-like signals but not activin/TGFβ-like signals and inhibits ciliated cell differentiation *in vitro*. In order to control *in vitro* differentiation of ciliated cells from ES cells, we tested the effect of a neutralizing antibody against BMP4. However, as shown in Fig. 2D, increasing concentration of this antibody failed to promote the ciliated cell differentiation in the presence of serum. We have also tested Noggin, a BMP antagonist, to increase the efficiency of the differentiation. But, no significant effect was observed [10].

Next, we decided to establish Tc-regulated ES cells expressing Smad7 or Smad6, both of which inhibit BMP signaling [19]. These Smad proteins are expressed only after depletion of Tc, indicating specific regulation of both inhibitory Smad proteins in our tet-off expression system (Fig. 3A). A green fluorescent marker protein, Venus, was designed such that it was coexpressed with Smad7 or Smad6 by the internal ribosome entry site (IRES) sequence. Using this system, the fluorescence of Venus can be used as a marker of Tc-regulated expression of Smad proteins (Fig. 3B). As expected, the ectopic expression of Smad7 or Smad6 significantly inhibited the phosphorylation of Smad1 (Fig. 3C).

Using these Tc-regulated ES cell lines, we next analyzed the effect of Smad7 and Smad6 on the ciliated-cell-marker gene expression under FBS-supplemented culture conditions. qRT-PCR analysis revealed that Smad7 markedly enhanced the expression of *Foxj1* mRNA, which is an essential gene for ciliogenesis (Fig. 4A). The expression of the gene encoding a component of cilia, *beta-tubulin IV*, and a ciliated cell-specific centriole-related gene, *centrin4* [20], were also upregulated in the Smad7-expressing cells. Induction of Smad6 also increased the expression of gene markers involved in ciliogenesis (Fig. 4B). Immunofluorescence analyses clearly revealed that induction of Smad7 dramatically promoted the differentiation of ES cells into beta-tubulin IV-positive ciliated cells (Fig. 4C) even in the presence of high concentration of FBS, which was shown to strongly inhibit differentiation of ES cells into ciliated cells. Similarly, induction of Smad6 also promoted ciliated cell differentiation (data not shown).

Interestingly, cilia on the Smad7-expressing cells cultured in FBS-containing medium were morphologically identical to those

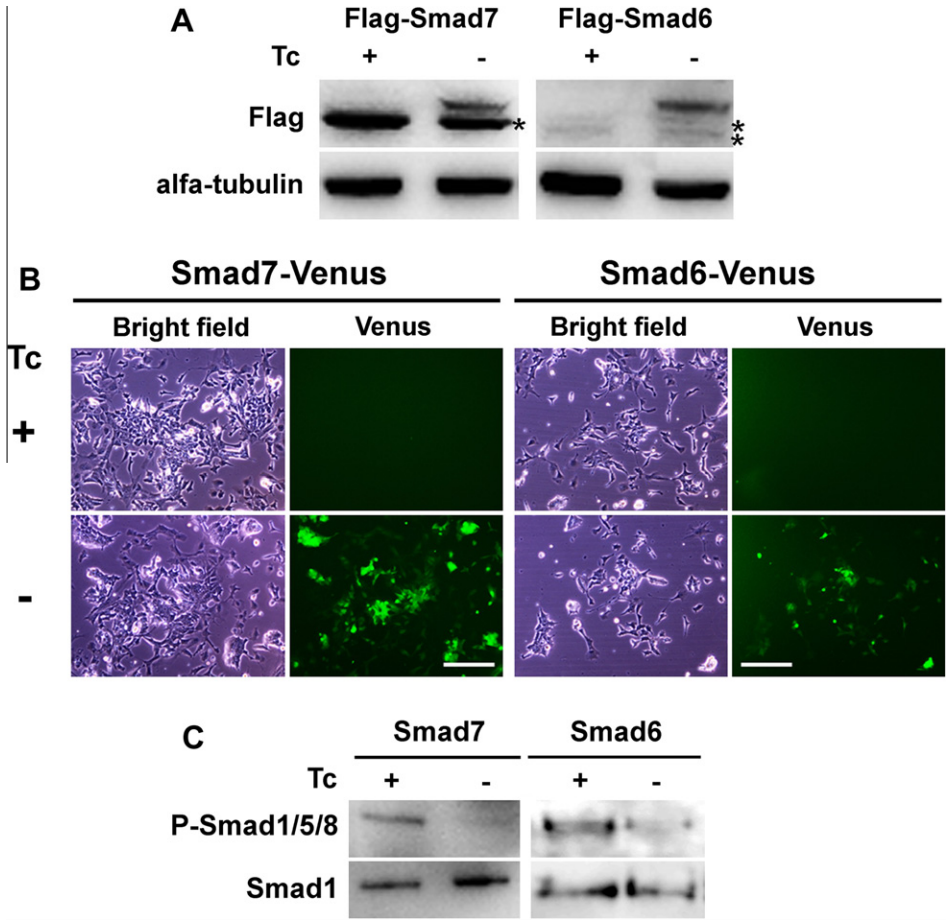

Fig. 3. Establishment of Tc-regulated Smad7- and Smad6-expressing ES cells. (A) Western blot analysis for Tc-dependent expression of Smad7 and Smad6. Expression of these Smad proteins was strictly regulated under the control of Tc. Asterisks indicate nonspecific bands. (B) Tc-dependent expression of a green fluorescent marker protein, Venus. In this system, Venus was coexpressed with Smad7 or Smad6 by the presence of the IRES sequence. Venus was expressed only in the absence of Tc. Scale bars; 200 μm. (C) Western blotting analysis for phosphorylated Smad1. Ectopic expression of Smad7 or Smad6 inhibited the phosphorylation of Smad1/5/8 in mouse ES cells.

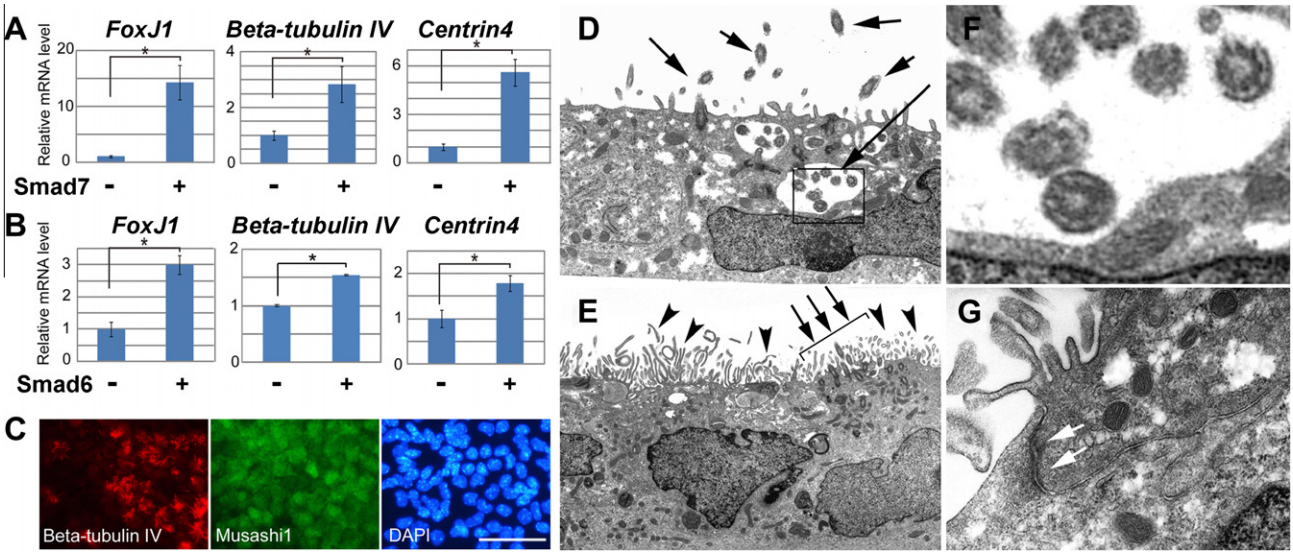

Fig. 4. Induction of ciliated cell differentiation in FBS-containing medium by the ectopic expression of Smad7 or Smad6. (A, B) Analysis of ciliated cell-marker gene expressions by qRT-PCR. Total RNA was extracted from EBs cultured for 15 days. The expressions of *Foxj1*, *beta-tubulin IV*, and *centrin4* mRNAs were all increased by the expression of either Smad7 (A) or Smad6 (B). Data are the mean ± SEM. The asterisks indicate a difference in means ($P < 0.05$). (C) Immunocytochemical analysis of beta-tubulin IV and Musashi1 expression in Smad7-expressing cells cultured in FBS-containing medium for 25 days. Ciliated cells induced by Smad7 were morphologically similar to type I ciliated cells and were positive for Musashi1. Scale bar; 50 μm. (D–G) Electron microscopy observation of induced ciliated cells by the ectopic expression of Smad7. (D) Low-magnification image of induced ciliated cells with sparse cilia (black arrows). (E) Ciliated epithelial cells covered by many microvilli (black arrow heads). The cilia projected from a localized area of the cell surface (black arrows). (F) Enlarged view of the cilia in the black square in (D) showing the typical 9 + 2 arrangement of microtubules. (G) These epithelial ciliated cells contact each other via tight junctions (white arrows).

on type I ciliated cells. Under these conditions, no type II ciliated cells were observed. Type I ciliated cell-specific differentiation was also confirmed by the expression of Musashi1 (Fig. 4C). Electron microscopy further revealed that these induced ciliated epithelial cells have a limited number of cilia projected from a localized area of cell surface (Fig. 4D–F). Many microvilli were also observed on the apical surface of ciliated cells (Fig. 4E). These ciliated cells contact each other via zonula adherens-type tight junctions (Fig. 4G). These characteristic properties of the induced ciliated cells resemble to those of ependymal cells [21,22]. Our results clearly indicate that inhibition of BMP-like activity by inhibitory Smad proteins significantly promotes ciliogenesis in mouse ES cells. Moreover, our data suggest that regulating BMP signaling with inhibitory Smad proteins could control cell type-specific differentiation of ciliated cells from stem cells.

4. Discussion

In this study, we identified inhibitory Smad proteins, Smad7 and Smad6, as important regulatory factors of ciliated cell differentiation from ES cells. We have demonstrated that inhibition of BMP signaling by increased expression of Smad7 or Smad6 in ES cells dramatically enhanced the expression of various ciliated cell-specific genes and eventually induced ciliated cell differentiation *in vitro*. On the other hand, a neutralizing antibody against BMP4 did not promote the differentiation of ES cells. Similarly, we could not observe significant effect of Noggin on the differentiation of ES cells into ciliated cells [10], which may be because Noggin can not inhibit all BMP-like activity in FBS. Noggin can effectively neutralize the activity of BMP2 and BMP4 but not those of other BMP family members such as BMP7 [23]. These results suggested that precise control of BMP-like activity is necessary to promote the ciliated cell differentiation.

Endogenous Smad7 and Smad6 are highly expressed in adult lung [24,25] and also expressed in embryonic lung during mouse development [26,27]. Although a majority of these signals are detected in the endothelial cells of the lung, the expression of Smad7 was observed in the bronchi of nascent lung [27]. Smad7 was also expressed in neuroepithelium of the forebrain and hindbrain [27]. These observations suggest that endogenous inhibitory Smad proteins might contribute to the differentiation of ciliated cells during lung and brain development.

We have also shown that ES cells cultured in serum-free medium differentiate into 2 types of ciliated cells (Fig. 1). Interestingly, ectopic expression of Smad7 or Smad6 protein induced differentiation of ES cells only into Musashi1-positive type I ciliated cells. Type II ciliated cells were not observed at all under these conditions. It is, therefore, plausible that BMP signaling is not only a crucial regulatory factor for the induction of ciliated cells, but also an important factor for cell-type specification of ciliated cells. Although we have not yet successfully determined the exact lineage specification of these ciliated cells, the expression of a ciliated ependymal cell-specific marker, Musashi1 [16], suggests a possibility that the type I ciliated cells could be ependymal cells. Electron microscopic analysis also revealed that the induced ciliated cells were morphologically similar to ependymal cells. Therefore, our results suggest that inhibitory Smad proteins could be novel regulatory molecules for the control of ciliated cell differentiation from pluripotent stem cells. Establishment of methods for specific ciliated cell differentiation from pluripotent stem cells into cells of the respiratory tract, reproductive organs, and brain ependyma could open new avenues in cell-based therapy for chronic bronchitis, sinusitis, or hydrocephalus in the near future.

Acknowledgments

We thank Dr. Shinji Masui, Dr. Kohei Miyazono, and Dr. Hideyuki Okano for providing us with the plasmids and antibodies. This work was supported in part by a grant from Wako Pure Chemical Industries, Ltd. (M.A.), a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Japan (M.A. and A.K.), a grant from the Project for Realization of Regenerative Medicine from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Japan (K.O.), a grant

from Ichiro Kanahara Foundation (A.K.), and a research fellowship from the Japan Society for the Promotion of Science for Young Scientists (Y.N.).

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