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Japan Elaprase® Treatment (JET) study: Idursulfase enzyme replacement therapy in adult patients with attenuated Hunter syndrome (Mucopolysaccharidosis II, MPS II)

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ABSTRACT

This open-label clinical study enrolled 10 adults with attenuated Mucopolysaccharidosis II and advanced disease under the direction of the Japan Society for Research on Mucopolysaccharidosis Disorders prior to regulatory approval of idursulfase in Japan. Ten male patients, ages 21–53 years, received weekly intravenous infusions of 0.5 mg/kg idursulfase for 12 months. Significant reductions in lysosomal storage and several clinical improvements were observed during the study (mean changes below). Urinary glycosaminoglycan excretion decreased rapidly within the first three months of treatment and normalized in all patients by study completion (−79.9%). Liver and spleen volumes also showed rapid reductions that were maintained in all patients through study completion (−33.2% and −31.0%, respectively). Improvements were noted in the 6-Minute Walk Test (54.5 m), percent predicted forced vital capacity (3.8 percentage points), left ventricular mass index (−12.4%) and several joint range of motions (8.1–19.0 degrees). Ejection fraction and cardiac valve disease were stable. The sleep study oxygen desaturation index increased by 3.9 events/h, but was stable in 89% (8/9) of patients. Idursulfase was generally well-tolerated. Infusion-related reactions occurred in 50% of patients and were mostly mild with transient skin reactions that did not require medical intervention. Two infusion-related reactions were assessed as serious (urticaria and vasovagal syncope). One patient died of causes unrelated to idursulfase. Anti-idursulfase antibodies developed in 60% (6/10) of patients. In summary, idursulfase treatment appears to be safe and effective in adult Japanese patients with attenuated MPS II. These results are comparable to those of prior studies that enrolled predominantly pediatric, Caucasian, and less ill patients. No new safety risks were identified.

Introduction

Mucopolysaccharidosis type II (MPS II, Hunter syndrome, OMIM #309900) is an X-linked recessive, lysosomal storage disorder caused by a deficiency of iduronate-2-sulfatase (IDS, EC3.1.6.13). This lysosomal enzyme catalyzes the first step in the degradation of the glycosaminoglycans (GAG), dermatan sulfate and heparan sulfate [1]. Iduronate-2-sulfatase deficiency leads to the accumulation of GAG within the lysosomes of virtually every cell in the body and is excreted in excessive amounts in the urine. MPS II encompasses a wide phenotypic spectrum that includes severe and attenuated forms. The severe form has onset of symptoms by 2–4 years old, progression of somatic symptoms and severe cognitive impairment during childhood, and death by 10–15 years of age. The attenuated form has a later onset in childhood, slower and milder progression of somatic disease, little to no cognitive impairment, and survival into adulthood. (Fig. 1) Common clinical features include coarse faces, upper airway obstruction, cardiac valve regurgitation, restrictive lung disease, hepatosplenomegaly, hernias, joint contractures, poor endurance, and reduced quality of life [2,3]. *IDS* gene mutations are heterogeneous, but some show genotype–phenotype correlations: deletions and gross rearrangements of the *IDS* gene are associated with the severe form, whereas missense mutations are more often associated with attenuated disease [4–10]. No racial or geographic differences have been observed. Females are only rarely affected, most often through skewed X-inactivation [1]. MPS II is the most prevalent MPS disorder in Asia, accounting for >50% of all MPS patients in Japan [10]. The annual incidence of all MPS disorders in Japan is estimated to be 1/50,000–1/60,000, and approximately half of the cases are due to MPS II. The estimated birth incidence of MPS II in Japan is, therefore, 1/90,000–1/100,000 [11], similar to the 1/92,000 to 1/162,000 incidences reported for predominantly Caucasian countries [12–15].

Until recently, treatment of MPS II was mainly palliative and focused on alleviating clinical symptoms through a variety of surgeries, medical devices, therapies, and medications. Several patients have undergone hematopoietic stem cell transplant (HSCT) as a source of iduronate-2-sulfatase, but unlike for MPS I, cognitive decline is not halted and the long-term effects on somatic disease are not well-documented [16,17]. Therefore, most centers consider the risk–benefit profile unfavorable and do not recommend HSCT for patients with MPS II.

Idursulfase (Elaprase®, Shire Human Genetic Therapies, Inc., Cambridge, MA, USA) is a recombinant human form of iduronate-2-sulfatase that is produced in a human cell line. Preclinical studies carried out in an MPS II knockout-mouse model [18] and in a Phase 1/2 dose-ranging study of MPS II patients [19] indicated that idursulfase was effective at reducing lysosomal GAG. The safety and efficacy of idursulfase was confirmed in a Phase 2/3 double-blind, placebo-controlled clinical study that randomized 96 MPS II patients to one of three treatment arms for 52 weeks: 0.5 mg/kg idursulfase weekly, 0.5 mg/kg idursulfase alternating with placebo every other week, or placebo weekly [20]. The primary efficacy endpoint was a composite of changes in percent predicted forced vital capacity (FVC) and the 6-Minute Walk Test (6MWT). Patients who received weekly idursulfase showed a greater difference in the composite endpoint compared to placebo ($p = 0.005$) than did the every other week idursulfase group ($p = 0.042$). The weekly idursulfase arm showed a mean 44.3 m increase in 6MWT distance (37 m difference from placebo, $p = 0.013$) and a mean 3.45 percentage point increase in percent predicted FVC (2.7 percentage point difference from placebo, $p = 0.065$). These clinical changes were associated with significant reductions versus placebo in urinary GAG level (−52.5%, $p < 0.0001$), liver volume (−25.3%, $p < 0.0001$), and spleen volume (−25.1%, $p < 0.0001$). Idursulfase was well-tolerated, with infusion-related reactions being the most common drug-related related adverse events, occurring in 69% (22/32) of patients in the weekly idursulfase arm.

Idursulfase was approved for the treatment of MPS II by the United States Food and Drug Administration (FDA) in July 2006 and by the European Medicines Agency (EMEA) in January 2007. Due to the life-threatening nature of the disease and the small number of patients, the Japanese Ministry of Health, Labour, and Welfare (MHLW) Committee for the Use of Unapproved Drugs recommended that idursulfase be approved based on ethical grounds and the results of overseas clinical trials, which included four Japanese patients. The committee also requested that idursulfase be made available to the most seriously ill MPS II patients prior to approval, which occurred in October 2007. Consequently, the Japan Elaprase Treatment (JET) study was initiated under the direction of the Japan Society for Research on MPS Disorders. Here, we present the results of this study.

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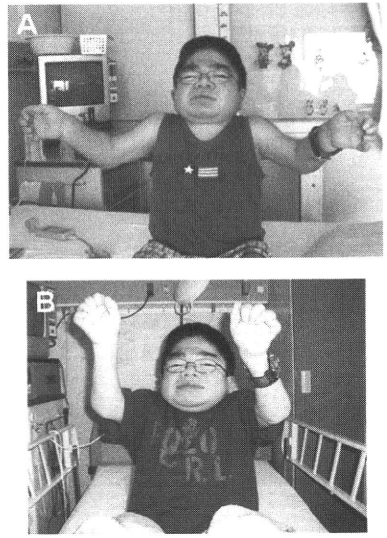

Fig. 1. A 23-year-old Japanese male study patient with MPS II. (A) Before treatment. (B) After 12 months of idursulfase treatment. Note the coarse facial features characteristic of MPS II. At baseline, the patient had severely limited shoulder range of motion (flexion and abduction), which improved following treatment.

Materials and methods

Patients

To be eligible for the study, patients had to meet all of the following inclusion criteria: (1) Documented deficiency of iduronate-2-sulfatase enzyme activity of <10% of the lower limit of normal with a normal enzyme activity level of one other sulfatase. (2) Male and above 20 years of age. (3) Clinically advanced disease status with <80% predicted FVC and New York Heart Association Class II–IV. (4) Capable of showing improved quality of life. (5) Able to complete study assessments.

Patient exclusion criteria included: (1) Previous bone marrow or cord blood transplant. (2) Known hypersensitivity to one of the components of idursulfase. (3) Previous treatment with idursulfase. (4) Unable to receive weekly infusions of idursulfase at the patient's local hospital. All patients provided signed informed consent prior to enrollment.

Study design

This was a multi-center, open-label study that enrolled 10 adult males with MPS II at 5 clinical sites in Japan. The study adhered to the guidelines set forth in the Declaration of Helsinki. Idursulfase was manufactured by Shire Human Genetic Therapies, Inc. and distributed by Genzyme Corporation (Cambridge, MA, USA). Genzyme Corporation performed all statistical analyses, and Genzyme Japan KK (Tokyo, Japan) provided data management support.

Idursulfase

Patients were administered 0.5 mg/kg idursulfase diluted in saline to a final volume of 100 cc intravenously over 3 h on a weekly

basis (±3 days) for up to 12 months. Infusions rates were ramped up over the first hour as described in the Phase 2/3 study [20]. Patients were monitored during each infusion and were discharged 1 h after completing the infusion, if clinically stable.

Efficacy assessments

Urinary GAG level was determined as the concentration of uronic acid normalized for creatinine (mg/g creatinine) and was measured using the carbazole reaction at a central laboratory (SRL Medisearch, Tokyo, Japan) or at Osaka City University Hospital. Liver and spleen volumes were quantitated by computerized tomography (CT), with the upper limits of normal being 2.5% and 0.2% of body weight, respectively. Percent predicted FVC and the 6MWT were performed according to American Thoracic Society guidelines [21,22]. Cardiac structure and function were evaluated by echocardiography (two-dimensional and M-mode). Left ventricular mass index (LVMI) was calculated as the left ventricular mass normalized for body surface area, with normal values defined as <131 g/m^2. Active joint range of motion was measured by goniometry, and included the shoulder (flexion, extension, and abduction), elbow (flexion and extension), hip (flexion and extension), and knee (flexion and extension). Left and right joint ranges of motion for each were averaged for each patient. The sleep study oxygen desaturation index (ODI) was assessed by pulse oximetry and defined as the number of desaturations (<89% oxygen saturation or ⩾4% decrease in oxygen saturation from baseline lasting ⩾10 s) per hour of sleep. A normal ODI was considered to be <5 events/h [23].

Safety assessments

Safety evaluation included continuous monitoring of adverse events and periodic clinical laboratory and physical examination evaluations. Adverse events were reported by severity (mild, moderate, severe, life-threatening) and by relatedness to idursulfase. An infusion-related reaction was defined as any adverse event occurring during or following an infusion (i.e., within 24 h of infusion initiation) that was reported by the investigator as related to idursulfase. Antibodies to idursulfase were measured by an enzyme-linked immunosorbant assay (ELISA; Shire Human Genetic Therapies).

Statistics

Efficacy results are reported as the mean ± standard error of the mean (SEM). For missing data at 12 months, the last observation carried forward method was used for values obtained at 6 months or later. The number of evaluable patients was at least nine for each endpoint, except for LVMI ($n = 6$, primarily due to missing baseline data) and the 6MWT ($n = 7$, primarily due to the inability to perform the test). The Wilcoxon signed rank test was used to evaluate changes in efficacy endpoint from baseline to 12 months, and p-values <0.05 were considered statistically significant. Percent change was tested for pharmacodynamic parameters (i.e., urinary GAG level and liver and spleen volumes), whereas absolute change was tested for clinical endpoints.

Results

Patient disposition

Ten adult Japanese males with attenuated MPS II were enrolled in the study and received idursulfase treatment. Nine patients completed the 12-month study; one patient died of causes unrelated to idursulfase after receiving 41 of 44 scheduled infusions (see Safety Section). Compliance with treatment was excellent, with all 10 patients receiving >93% of scheduled infusions; 80% (8/10) of patients did not miss a single scheduled infusion.

Patients

The mean patient age was 30.1 years (range 21.1–53.9). All patients had been diagnosed during mid-childhood or adolescence with MPS II (mean age 7.9 years), and all had advanced disease burden at the time of enrollment into the study. All patients had short stature (height <3rd percentile for Japanese adult males). Past medical history was significant for the following MPS II-related features (n = number of patients): valvular heart disease consisting mainly of aortic and/or mitral valve insufficiency (10), joint contractures (7), hepatomegaly (7), deafness (6), retinal degeneration (5), sleep apnea (5), otitis media

Table 1
Summary of efficacy changes after 12 months of treatment with idursulfase.

	N	Baseline	12 months	Change	% Change	p-Value
Urinary GAG (mg/g creatinine)	9	106.4 ± 7.8	21.2 ± 2.9	−85.2 ± 7.1	−79.9 ± 2.2	0.004[†]
Liver volume (cc)	10	1491.2 ± 92.9	993.2 ± 75.0	−498.0 ± 70.2	−33.2 ± 4.0	0.002[†]
Spleen volume (cc)	10	210.2 ± 22.5	138.1 ± 12.5	−72.1 ± 15.7	−31.0 ± 5.5	0.002[†]
6-Minute Walk Test (m)	7	286.0 ± 53.4	340.5 ± 49.6	54.5 ± 27.0	37.4 ± 18.1	0.109
Forced vital capacity (% predicted)	9	39.9 ± 6.6	43.7 ± 6.0	3.8 ± 2.8	15.0 ± 8.0	0.250
Forced vital capacity (L)	9	1.4 ± 0.3	1.5 ± 0.2	0.1 ± 0.1	16.3 ± 8.0	0.250
Left ventricular mass index (g/m^2)	6	139.9 ± 25.1	133.2 ± 38.9	−6.7 ± 15.5	−12.4 ± 11.1	0.563
Left ventricular ejection fraction (%)	10	67.0 ± 5.2	64.3 ± 6.0	−2.8 ± 2.5	−6.1 ± 5.7	0.244
Joint range of motion (degrees)					NA	
Shoulder flexion	10	93.8 ± 4.9	109.8 ± 7.1	15.0 ± 7.3		0.066
Shoulder extension	10	44.1 ± 4.1	43.8 ± 3.8	−0.3 ± 4.1		0.945
Shoulder abduction	10	76.3 ± 3.9	95.3 ± 8.1	19.0 ± 8.8		0.125
Knee flexion	9	103.7 ± 8.5	114.4 ± 5.2	10.7 ± 10.3		0.461
Knee extension	9	−11.1 ± 4.5	−10.3 ± 5.0	0.8 ± 2.5		0.875
Hip flexion	9	89.2 ± 8.1	103.3 ± 7.6	14.2 ± 5.1		0.031
Hip extension	9	3.1 ± 5.0	1.9 ± 6.7	−1.3 ± 1.8		0.750
Elbow flexion	10	120.9 ± 4.0	121.8 ± 3.7	0.9 ± 2.5		0.828
Elbow extension	10	−43.1 ± 4.2	−35.0 ± 4.2	8.1 ± 3.4		0.063
Oxygen desaturation index (events/h)	9	18.5 ± 6.1	22.3 ± 7.4	3.9 ± 3.5	NA	0.426

The last observation carried forward (LOCF) method was used to replace a missing value at the 12-month timepoint.
All values are the observed means ± SEM. All p-values are based on the Wilcoxon signed rank test for change from baseline to the 12-month timepoint.
NA, not applicable. Some patients had values of 0 at baseline that precluded calculation of percent change.
[†] The p-value is based on the Wilcoxon signed rank test for % change from baseline to the 12-month timepoint.

(4), macroglossia (3), umbilical hernia (2), carpal tunnel syndrome (2), heart failure (2), and left ventricular hypertrophy (1).

Urinary glycosaminoglycan (GAG)

All nine evaluable patients had elevated urinary GAG levels at baseline (mean 106.4 mg/g creatinine, approximately 8 times the upper limit of normal); one patient lacked an appropriate baseline value (Table 1). Following idursulfase treatment, urinary GAG levels decreased rapidly within the first three months of treatment and remained low for the remainder of the study (Fig. 2A). There was a statistically significant mean decrease in the urinary GAG level of −79.9 ± 2.2% from baseline to 12 months ($p = 0.004$). All nine evaluable patients showed a >70% decrease in urinary GAG levels and had normal values by the end of the study.

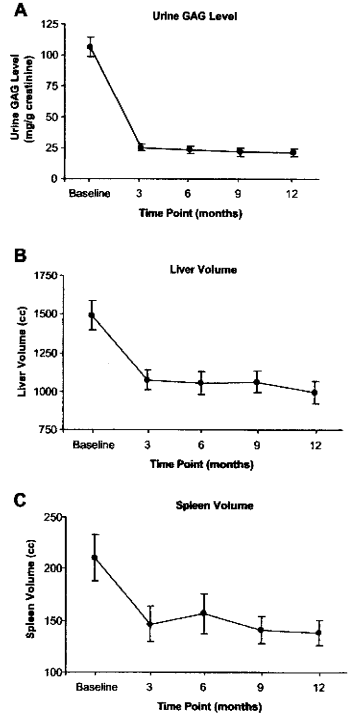

Fig. 2. The effects of idursulfase treatment on lysosomal storage over 12 months. (A) Urinary GAG level. (B) Liver volume. (C) Spleen volume. All changes are reported as mean ± SEM.

Liver and spleen volumes

At baseline, 9 (90%) patients had hepatomegaly (mean 1.3 MN, multiples of normal) and all 10 (100%) patients had splenomegaly (mean 2.4 MN) by CT. After 12 months of treatment, mean liver volume decreased by −33.2 ± 4.0% and mean spleen volume decreased by −31.0 ± 5.5% (Fig. 2B and C; Table 1), and both changes were statistically significant ($p = 0.002$). Most of the reductions occurred within the first three months of treatment. By the end of the study, all patients had liver volumes within the normal range and spleen volumes that were <2 MN, demonstrating efficient reduction of lysosomal GAG storage.

6-Minute Walk Test (6MWT)

At baseline, the mean 6MWT distance was 286.0 m for the seven patients who could perform the test (Table 1). All but one patient walked <399 m, the lower limit of normal for healthy adult men in the United States [24]. Three patients could not perform the 6MWT: one patient broke his leg just prior to the start of the study; one patient was wheelchair-bound secondary to shortness of breath and muscle weakness; and one patient was obese and could only walk a few steps with assistance. By the end of the study, the mean 6MWT distance had increased by 54.5 ± 27.0 m (Fig. 3A). This change represents a relative increase of 37.4%, and included one patient whose 6MWT distance increased by 131%. Four patients (57%) showed a clinically meaningful improvement of ⩾54 m [25], while the one patient with a normal 6MWT at baseline showed a decline (−71 m).

Percent predicted forced vital capacity (FVC)

Nine patients underwent spirometry at baseline and all showed a restrictive lung disease pattern: three were classified as having a severe defect (<50% predicted FVC) and five had a very severe defect (<34% predicted FVC) [26]. At baseline, mean percent predicted FVC was 39.9% (Table 1), and after 12 months it increased by 3.8 ± 2.8 percentage points (Fig. 3B). This improvement corresponds to a relative increase of 15.0% over baseline, which is considered clinically meaningful (⩾15% relative change) [25] and was achieved by four (44%) patients. Similarly, mean FVC increased by 16.3% over the baseline of 1.4 L. The mean forced expiratory volume in 1 s (FEV_1):FVC ratio remained unchanged at 0.70 during the study.

Cardiac

All patients had valve disease that remained stable during the study. The mean ejection fraction (EF) was normal at baseline and showed little change over 12 months (67.0–64.3%, change of −2.8 ± 2.5%) (Table 1). One patient with pre-existing cardiac failure showed gradual worsening during the study (EF 27–14%). At baseline, mean LVMI was slightly elevated at 139.9 g/m^2 (normal <131 g/m^2), and 50% (3/6) of evaluable patients had an elevated LVMI. After 12 months, mean LVMI decreased by −12.4%, with four patients showing a clinically meaningful improvement of >10% [27]. The patient with the largest LMVI at baseline showed a further increase (254.1–312.9 g/m^2).

Joint range of motion

Fig. 4 and Table 1 show the changes in joint range of motion observed during the study. At baseline, patients had significant joint contractures involving the shoulder (flexion, extension, and abduction), knee (flexion and extension), hip flexion and extension), and elbow (flexion and extension). Following 12 months of treatment, several joints showed increased range of motion, including mean

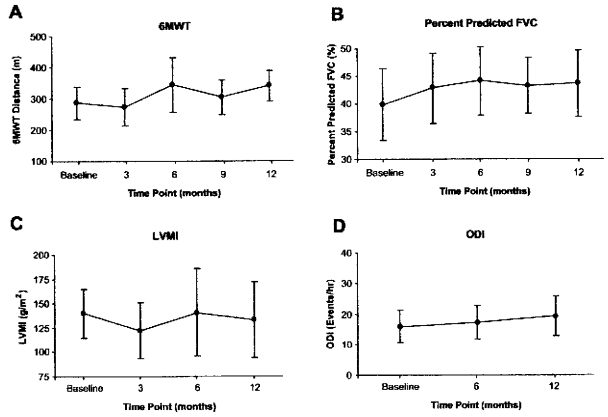

Fig. 3. The effects of idursulfase treatment on clinical endpoints over 12 months. (A) 6-Minute Walk Test. (B) % Predicted forced Vital Capacity. (C) Left Ventricular Mass Index. (D) Oxygen Desaturation Index. All changes are reported as mean ± SEM.

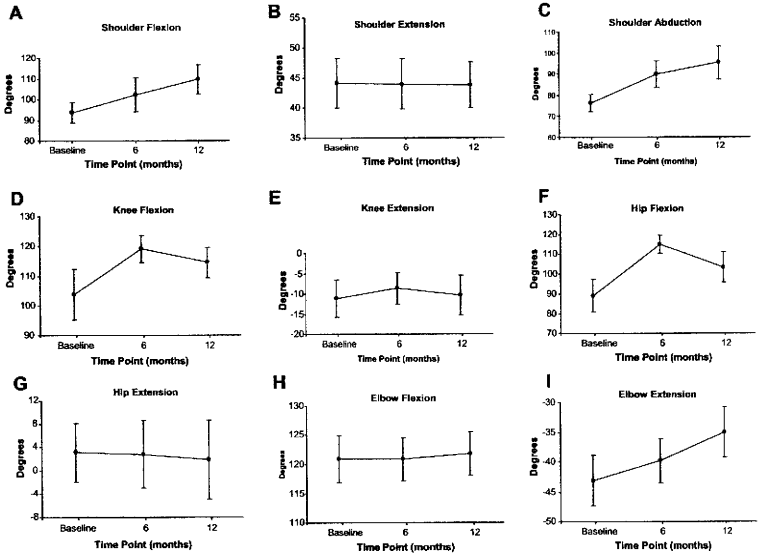

Fig. 4. The effects of idursulfase treatment on joint range of motion over 12 months. (A) Shoulder flexion. (B) Shoulder extension. (C) Shoulder abduction. (D) Knee flexion. (E) Knee extension. (F) Hip flexion. (G) Hip extension. (H) Elbow flexion. (I) Elbow extension. All changes are reported as mean ± SEM.

shoulder flexion (15.0 ± 7.3 degrees), shoulder abduction (19.0 ± 8.8 degrees), knee flexion (10.7 ± 10.3 degrees), hip flexion (14.2 ± 5.1 degrees; $p = 0.031$), and elbow extension (8.1 ± 3.4 degrees). However, most of the changes did not achieve statistical significance. Shoulder extension (−0.3 ± 4.1 degrees), elbow flexion (0.9 ± 2.5 degrees), knee extension (0.8 ± 2.5 degrees), and hip extension (−1.3 ± 1.8 degrees) showed little change during the study. Fig. 1 shows a 23 year-old study patient with severely limited shoulder range of motion (abduction and flexion), which improved following one year treatment with idursulfase.

Oxygen desaturation index (ODI)

At baseline, the mean oxygen desaturation index (ODI) was 18.5 events/h ($n = 9$), which is moderately abnormal [23]. Three patients had a normal ODI (<5 events/h), two had a mildly abnormal ODI (5–15 events/h), and four had a moderately to severely abnormal ODI (>15 events/h). During the study, the mean ODI increased by 3.9 ± 3.5 events/h, which was largely due to a single patient with an increase of 26.8 events/h. The other seven patients had stable ODI values (changes ≤ 10 events/h).

Safety

Idursulfase was well-tolerated over the course of the study. Adverse events were mainly mild, unrelated, and attributable to expected symptoms of MPS II disease. Fifty percent (5/10) of patients experienced a total of 11 drug-related adverse events. Urticaria was the most frequent event (five events in two patients), followed by erythema (two events in the same patient). Similarly, 50% (5/10) of patients experienced infusion-related reactions (i.e. adverse events assessed as drug-related and occurring within 24 h of the infusion). The highest patient incidence involved skin reactions, i.e. urticaria and erythema (three patients each), while dyspnea, abdominal pain, and vasovagal syncope also were observed in one patient each. Except for one patient who experienced several episodes of urticaria between 9 and 12 months, the other four patients had infusion-related reactions only once or twice during the first three months of treatment. Management of infusion-related reactions included antihistamine therapy and temporary interruption of the infusion, and all events were followed by a successful patient recovery. There were no clinical laboratory abnormalities reported as related to idursulfase.

Two patients experienced serious adverse events, including one death, in the study. A 26 year-old male experienced an infusion-related reaction involving diffuse urticaria, flushing, and numbness of the tongue 1 h after initiation of the fifth infusion. The patient was pre-medicated with antihistamines without further events. A 42 year-old male had an infusion-related reaction reported by the investigator as vasovagal syncope, which consisted of hypotension, vomiting, weak pulse, and decreased consciousness and occurred 30 min into the first infusion. Subsequent infusions were preceded by corticosteroid pre-medication administration without further infusion-related reactions. The patient had a history of cardiac valve incompetence and cardiac failure requiring medications, including furosemide. Later in the study, he experienced an increase in leg edema secondary to worsening congestive heart failure. He was depressed and attempted suicide by drug overdose (not idursulfase). Upon arrival at the hospital, the patient went into cardiac arrest. Subsequent resuscitation measures were unsuccessful, and he died due to hypoxic encephalopathy, pneumonia and renal failure.

Antibodies

Anti-idursulfase IgG antibodies were detected in 60% (6/10) of patients, two of who became seronegative later in the study. No IgE antibodies were detected in patients who underwent testing for infusion-related reactions. The mean reductions in urinary GAG levels did not differ between patients who were seropositive at any time (−80.9% ± 3.8%; $n = 5$) and those who remained seronegative throughout the study (−78.6% ± 1.8%; $n = 4$). Although hypersensitive reactions or infusion-related adverse reactions tended to occur in the antibody-positive patients (four antibody-positive patients versus one antibody-negative patient), there was no correlation between the presence of antibodies and other adverse events. Furthermore, the frequency of hypersensitivity reactions did not correlate with antibody titer.

Discussion

The most remarkable difference between this and previous clinical studies of idursulfase [19,20] relates to the patient demographics and characteristics. The purpose of the JET study was to provide access to treatment for the most seriously ill MPS II patients while awaiting regulatory approval of idursulfase in Japan, which occurred in October 2007. Patients in the JET study had a mean age of 30.1 years, all were Japanese, and all were seriously ill (mean percent predicted FVC 39.9% and mean 6MWT distance 286.0 m). By comparison, MPS II patients in the Phase 1/2 and Phase 2/3 studies of idursulfase were younger (mean ages 13.9 years and 14.2 years), predominantly Caucasian (100% and 83%, respectively), and less severely affected (mean percent predicted FVC 55.1% and 55.4%; mean 6MWT distance 397 m and 395 m) [19,20]. Despite these patient differences, the JET study has shown that idursulfase is a safe and effective (Table 1) treatment for Japanese patients with MPS II and its risk–benefit profile is similar to that reported in previous studies.

In this study, idursulfase efficiently reduced GAG storage, as evidenced by the statistically significant reductions in urinary GAG levels ($p = 0.004$) and hepatosplenomegaly ($p = 0.002$) (Fig. 2; Table 1). These pharmacodynamic changes appeared to translate into clinical benefit, as evidenced by trends towards improvement in functional capacity (mean 54.5 m increase in 6MWT), respiratory function (mean 15.0% relative increase in percent predicted FVC), joint range of motion (mean increases ranging from 8.1–19.0 degrees for several joints), and LVMI (mean −12.4% decrease). Cardiac EF and valve disease remained mostly stable, although one patient with severe congestive heart failure showed progressive worsening and one patient with a greatly elevated LVMI showed a further increase. The mean ODI increased slightly by 3.9 events/h, but importantly 89% (8/9) of patients showed no clinically significant changes.

The safety profile of idursulfase in the JET study was similar to that of previous studies with no new or unexpected adverse events despite the older and more seriously ill patient population. Most adverse events were considered by investigators to be disease-related and unrelated to idursulfase. The most common drug-related adverse events were infusion-related reactions, occurring in 50% of patients. The most common infusion-related reactions were skin reactions consisting of urticaria and erythema. There were two related serious adverse events that occurred during the infusions—one involving urticaria, flushing, and numbness of the tongue, and the other involving vasovagal syncope. The one patient death was attributed to suicide from a drug overdose and was not related to idursulfase.

MPS II is a progressive and debilitating multisystem disease that is associated with a shortened lifespan, primarily from cardiorespiratory compromise [28]. Therefore, it is noteworthy that in this one-year study, cardiac and respiratory functions were improved or stable in most patients. Decreasing lung volumes are known to be associated with increased morbidity and mortality [26];

given the low percent predicted FVC values at baseline in study patients (mean 39.9%), a relative increase of 15% is of particular importance. The American Thoracic Society defines a >15% relative change in FVC occurring over a one-year period as being clinically meaningful [26]. Similarly, the 54.5 m mean increase in 6MWT distance also is considered to be a clinically meaningful improvement, based on a study of adult men with chronic obstructive pulmonary disease [25]. The 6MWT is a sub-maximal exercise test that is a composite assessment of cardiac, respiratory, and musculoskeletal function. Because all three of these organ systems are involved in the MPS disorders, walking tests have been widely used as primary efficacy endpoints in clinical trials of enzyme replacement therapy for other MPS disorders, including MPS I [29,30] and MPS VI [31].

We observed no evidence for an effect of race on immunogenicity or safety. IgG antibodies were detected in 60% (6/10) of patients treated with idursulfase, which is similar to the 49.6% rate seen in the Phase 2/3 study that enrolled predominantly Caucasian and other non-Asian patients [20]. In addition, the adverse event profile was similar in all respects; infusion-related reactions occurred in 50% of patients in the current study compared to 69% of patients receiving weekly idursulfase in the Phase 2/3 study [20].

Limitations of this study include its open-label treatment, lack of control group, and small sample size. Other aspects of the study design, however, including the treatment dose and regimen, study duration, and efficacy and safety assessments were identical or very similar to those used in the Phase 2/3 study [20]. A placebo effect in this study cannot be excluded, especially for effort-dependent assessments such as the 6MWT and active joint range of motion. Nevertheless, the magnitude of change in the 6MWT distance was similar to those observed in previous studies of idursulfase [19,20]. Determination of FVC by spirometry is less susceptible to a placebo effect given the requirement for test–retest reproducibility at each assessment [21]. This study enrolled only 10 patients, which may not have had sufficient power to detect a statistically significant clinical response even if clinical improvements were present. On the other hand, the biomarkers of lysosomal GAG clearance, i.e. liver and spleen volumes and urinary GAG level, did have sufficiently large effect sizes (change/standard deviation of change) to show statistically significant differences. Finally, the study involved only adult males, all of whom had a substantial pre-existing disease burden. This study shows that many disease features of seriously ill patients, including diminished cardiorespiratory function, restricted joint range of motion, and hepatosplenomegaly can improve with idursulfase treatment. An even better response is expected in young children prior to final organ maturation and the development of chronic tissue damage. In this regard, a study in MPS II patients ⩽5 years of age is underway.

Conclusions

Idursulfase was generally well-tolerated and produced clinical improvements in adult Japanese patients with attenuated MPS II treated with the labeled dose, 0.5 mg/kg administered intravenously once weekly. Treatment with idursulfase also resulted in substantial reductions in hepatosplenomegaly and urinary GAG excretion, indicating efficient clearance of lysosomal GAG. The safety profile and immunogenicity of idursulfase appear to be similar between Japanese and previously studied Caucasian patients.

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Short communication

Cervical pachymeningeal hypertrophy as the initial and cardinal manifestation of mucopolysaccharidosis type I in monozygotic twins with a novel mutation in the alpha-l-iduronidase gene

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ABSTRACT

We describe a pair of monozygotic twins with an attenuated form of mucopolysaccharidosis type I (MPS-I). At age 24, they both developed cervical myelopathy as a cardinal manifestation. They each also had mild valve abnormalities and both inguinal and umbilical hernia, however, other characteristic features of MPS-I were absent or very mild. Magnetic resonance imaging revealed the cervical cord compressed by pachymeningeal hypertrophy. Surgery with dural plasty and laminoplasty resulted in decompression of the cervical cord with clinical improvement, revealing marked thickening of the dura mater. Both patients showed a marked decrease of alpha-l-iduronidase (IDUA) activity with c.252insC (p.P55fsX62; known) and c.1209C>A (p.T374N; novel) mutations of the IDUA gene (*IDUA*). Patients with MPS-I have been reported to present with various clinical phenotypes and severities even if they have identical mutations of *IDUA*. The quite similar, unique phenotype in monozygotic twins suggests that not only *IDUA* mutation but also other genetic factors than *IDUA* markedly influence the clinical manifestations of MPS-I.

1. Introduction

Mucopolysaccharidosis type I (MPS-I) is an autosomal recessive disorder caused by a deficiency of α-L-iduronidase (IDUA), which leads to the accumulation of dermatan sulfate and heparan sulfate throughout the body. The IDUA gene (*IDUA*) is located on chromosome 4p 16.3 containing 14 exons [1], and approximately 100 mutations of *IDUA* have been identified [2]. MPS-I presents with various clinical manifestations including valve abnormality, joint contracture, corneal clouding, and course facial features [3]. Some patients with MPS-I have been reported to show cervical myelopathy due to cervical pachymeningeal hypertrophy during their clinical course [4,5]; however, cervical myelopathy is rare as the initial and cardinal manifestation of MPS-I.

We describe monozygotic twins with an attenuated form of MPS-I associated with a novel mutation of *IDUA*, who both showed cervical myelopathy as the initial and cardinal manifestation.

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2. Case report

2.1. Patient 1

A 24-year-old Japanese man was admitted because of a 6-month history of progressive gait disturbance. He was born from non-consanguineous, young and healthy parents. He had a healthy elder brother and an affected twin brother (Patient 2). Pregnancy and labor had been uneventful, and birth weight was 2300 g. Mental and motor development were normal, and he had graduated from a vocational school. Past medical histories included Kawasaki disease at age 6 months, and inguinal hernia treated by surgical repair in childhood and again at age 20.

Physical examination demonstrated a height of 156.6 cm (mean height of Japanese male at age 24 is 170.9 ± 6.0 (SD) cm according to the National Health and Nutrition Survey in Japan, 2006), body weight of 45.8 kg (mean body weight of Japanese male at age 24 is 62.6 ± 9.8 (SD) kg according to the National Health and Nutrition Survey in Japan, 2006), systolic ejection heart murmur and umbilical hernia. Joint contractures, enlargements of tonsils and tongue, hepatomegaly and splenomegaly were all absent. Corneal clouding evaluated by ophthalmologist with slip lamp was also absent, and the facial features were normal. On neurologic examination, both legs were markedly spastic with a scissors gait. Deep tendon reflexes were mildly exaggerated in the upper extremities, and markedly exaggerated in the lower extremities with bilateral Babinski signs.

Findings on urinalysis, routine hematological and blood chemistry examinations were all normal. Urine chemistry examination demonstrated increased excretion of uronic acid (63.1 mg/g creatinine; normal range 8.3–12.3 mg/g creatinine). Electrocardiogram was normal, and nerve conduction studies (NCS) on bilateral median nerve showed no evidence of carpal tunnel syndrome. Radiography of the chest demonstrated mild thoracic deformity (Fig. 1A) and that of cervical spine demonstrated hypoplasia of vertebral body and spinous process (Fig. 1B). Transthoracic echocardiogram (TTE) demonstrated mild aortic valve stenosis (1.2 cm^2 of average valve area (AVA)) with pressure gradient (PG) across the aortic valve of 33.6 mm Hg with normal wall motion of the left ventricle. Magnetic resonance image (MRI) of the cervical spine showed severe cervical cord compression due to thickening of the surrounding dura mater without gadolinium enhancement at C2-6 levels (Fig. 2A, C, E). Brain MRI demonstrated enlarged perivascular space and small hyperintense lesions on fluid attenuated inversion recovery image (Fig. 3A, C, E). There is no dural thickening in intracranial, thoracic, and lumbar region. Cerebrospinal fluid (CSF) examinations demonstrated an elevated protein level (440 mg/dL; normal range 10–40 mg/dL), which would be resulted from CSF circulatory disturbance caused by severe spinal canal stenosis, without pleocytosis. Cytology and culture for bacteria and mycobacteria were negative. Wechsler Adult Intelligence Scale Third Edition (WAIS-III) demonstrated overall intelligence quotient (IQ) of 101, verbal IQ of 93 and performance IQ of 112.

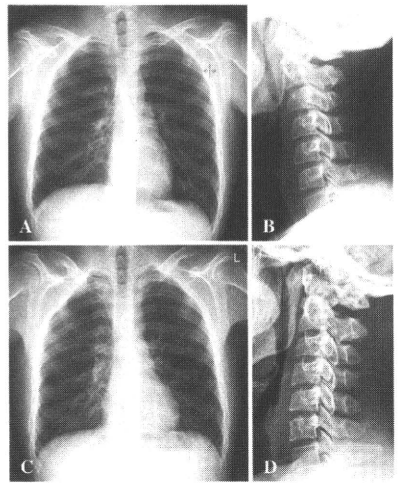

Fig. 1. Radiographies of the chest and cervical spine of Patients 1 (A, B) and 2 (C, D) showed mild thoracic deformity (A, C), and mild hypoplasia of vertebral body and spinous process (B, D).

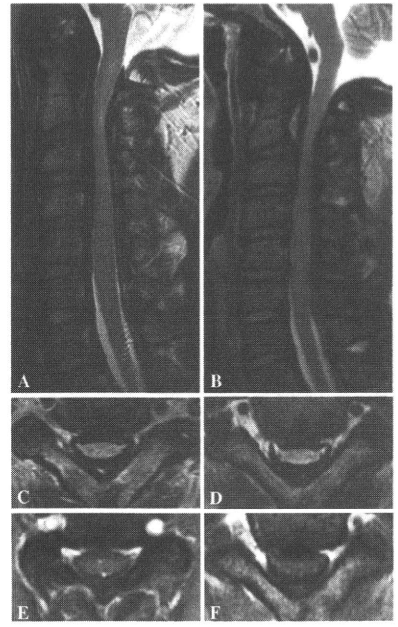

Fig. 2. MRI of the cervical spine of Patients 1 (A, C, E) and 2 (B, D, F) showed severe cord compression at C2-6, surrounded by thickened dura mater. The thickened dura was characterized by hypointensities on both T1- and T2-weighted images, but there was no gadolinium enhancement (A–D: T2-weighted image; E, F: T1-weighted image with gadolinium enhancement; C–F: axial sections at C2/3 level).

2.2. Patient 2

A 24-year-old man, the twin brother of Patient 1, was admitted to our ward because of a 3-month history of progressive gait disturbance. Onset was 6 months after the development of gait disturbance in Patient 1. Birth weight was 1900 g and mental and motor development were both normal. He had graduated from high school. Past medical histories included inguinal hernia treated by surgical repair at 2 months of age and bronchial asthma since 4 years of age.

Physical examination demonstrated a height of 157.0 cm, body weight of 45.3 kg, systolic ejection heart murmur, umbilical hernia, and minor joint contractures of the elbow and knee. Enlargements of tonsils and tongue, hepatomegaly and splenomegaly were all absent. Corneal clouding evaluated by ophthalmologist with slip lamp was also absent, and the facial features were normal. On neurologic examination, both legs were markedly spastic with spastic gait. He had mild hypoesthesia below the sensory level around C5. Romberg sign was positive. Exaggeration of deep tendon reflexes was slight in the upper extremities, and marked in the lower extremities with bilateral Babinski signs.

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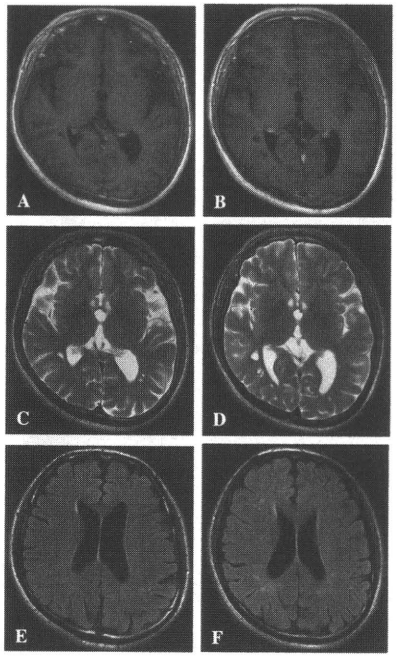

Fig. 3. Brain MRI of Patients 1 (A, C, E) and 2 (B, D, F) showed enlarged perivascular space around the posterior horn of the lateral ventricle (A–D), and small hyperintense lesions in deep white matter (E, F). There is no thickening and abnormal enhancement of the intracranial dura mater (A, B: T1-weighted image with gadolinium enhancement; C, D: T2-weighted image; E, F: fluid attenuated inversion recovery image).

Urinalysis, routine hematological and blood chemistry examinations all showed normal findings. Urine chemistry examination demonstrated increased excretion of uronic acid (51.7 mg/g creatinine). Radiographies of chest and cervical spine showed similar findings to those in Patient 1 (Fig. 1C, D). Electrocardiogram showed increased amplitude of QRS complex without ST changes. NCS on bilateral median nerve did not show any abnormal findings. TTE demonstrated mild aortic valve stenosis (1.3 cm^2 of AVA) with PG across the aortic valve of 33.6 mm Hg with normal wall motion of the left ventricle. MRI of the cervical spine and brain showed findings quite similar to those in Patient 1 (Figs. 2B, D, F and 3B, D, F). Brain, thoracic, and lumbar MRI did not show any dural thickening. CSF examinations demonstrated an elevated protein level (342 mg/dL) without pleocytosis. WAIS-III demonstrated an overall IQ of 75, verbal IQ of 67 and performance IQ of 90.

Both patients underwent dural plasty with laminoplasty to decompress the cervical spinal cord, and the surgery resulted in obvious improvement of spastic gait. Biopsies of the dura mater during the surgery disclosed marked thickening of the collagenous tissue with clear cells containing fine granular materials stained by Alcian-Blue, which is presumably lysosomal inclusions of accumulated glycosaminoglycan in both patients (Fig. 4). Furthermore, both patients initiated enzyme replacement therapy with laronidase, which expected to decrease glycosaminoglycan storage and lead to clinical improvement [6], for MPS-I after surgery.

3. Methods

3.1. Enzymatic assay of IDUA activity

Enzymatic activity of IDUA from peripheral blood leukocytes was measured by fluorometric assay using 4-methylumbelliferyl α-L-iduronide as described previously [7].

3.2. Zygosity testing of the twin

Determination of zygosity was made by PCR-amplified short tandem repeat (STR) analysis [8] with commercially available panels (AmpFLSTR SGM Plus Kit and AmpFLSTR Profiler Kit, Applied Biosystems LLC, Foster City, CA, USA), comprising 15 autosomal, codominant, unlinked loci (D3S1358, vWA, FGA, D16S539, D2S1338, TH01, TPOX, CSF1PO, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820, D19S433), and the gender-determining marker, amelogenin.

3.3. Mutation analysis of the IDUA gene

Genomic DNA was extracted from peripheral blood leukocytes. PCR amplification of each of 14 exons and intron/exon boundaries of *IDUA* was performed as reported in a previous publication [9]. PCR products were sequenced in both the forward and reverse directions on automated DNA sequencing (ABI Prism 3100 Genetic Analyzer) using the BigDye Terminator v3.1 Cycler Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA).

4. Results

4.1. Enzymatic assay of IDUA activity

Both patients showed IDUA activity from peripheral leukocytes <0.9 nmol/mg protein/h (normal range; 29.8–89.8 nmol/mg protein/h), and that of their mother showed 19.9 nmol/mg protein/h.

4.2. Zygosity testing of the twin

All 16 tested loci and marker were identical in our patients, therefore, the patients were estimated to be monozygotic twins with probability of 99.99995% (based on the assumption of an a priori probability at 0.5).

4.3. Mutation analysis of the IDUA gene

Direct sequence from genomic DNA of the twins showed 1 bp insertions, c.252insC (p.P55fsX62), in exon II, and point mutations, c.1209C>A (p.T374N), in exon VIII of *IDUA* in a heterozygous form in the two patients (Fig. 5). These mutations were examined in their mother, who showed only a heterozygous form of the mutant and a wild type sequence for c.252insC (data not shown).

5. Discussion

We report two patients with an attenuated form of MPS-I confirmed by *IDUA* mutations. It was characteristic that these patients showed a quite similar distinctive phenotype: development of cervical myelopathy due to spinal canal stenosis caused by pachymeningeal hypertrophy as an initial and cardinal manifestation at the same age. In addition, our patients are short compared with their same generation of Japanese male, and have a history of umbilical and inguinal hernia, mild cardiac valve abnormality, dysostosis multiplex and joint contracture (in Patient 2 only), which are likely MPS-I-related manifestations. Importantly, genetic analyses demonstrated that our patients were monozygotic twins showing the identical mutations of *IDUA*, c.252insC (p.P55fs62X) and c.1209C>A (p.T374N). Although we could not perform *IDUA* analysis of the father, the mother showed only c.252insC mutation. Therefore, the c.1209C>A mutation was inferred to have been inherited from their father, and the patients would have compound heterozygous mutations.

MPS-I has been historically classified into three phenotypes, severe (Hurler), attenuated (Scheie), and intermediate (Hurler/Scheie); however, the clinical spectrum is variable from severe to mild [10]. The clinical features of our patients related to MPS-I are generally mild, and age at diagnosis is relatively late, therefore, we consider our patient as having attenuated form of MPS-I. Our patients each had a history of inguinal and umbilical hernia in early childhood and cervical myelopathy developed two decades after surgical treatment of hernias. This time course of our patients appears to fit the natural history of attenuated form of MPS-I [11]. Cardiac valve abnormalities, joint contracture, corneal clouding, carpal tunnel syndrome, and hernia were reported to be the five most prevalent features of attenuated form of MPS-I [11]. Although our patients each had some features of attenuated MPS-I, they showed no corneal clouding, carpal tunnel syndrome with no or very

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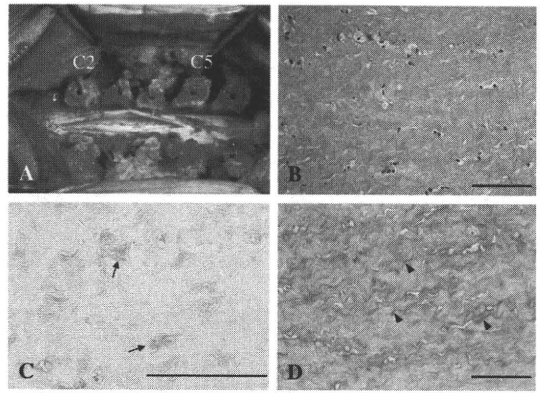

Fig. 4. Surgical and pathological findings of the dura mater of Patient 2. Thickened dura was observed macroscopically (A). Cervical dural biopsy showed clear cells containing fine Alcian-Blue-positive granular materials, which is presumably indicating lysosomal deposit of glycosaminoglycan, within the cytoplasm (arrows), and Periodic acid-Schiff (PAS)-positive deposits between collagenous connective tissue (arrow heads) (B–D) (B: Hematoxylin–eosin stain, C: Alcian-Blue stain, D: PAS stain, scale bar indicates 100 μm).

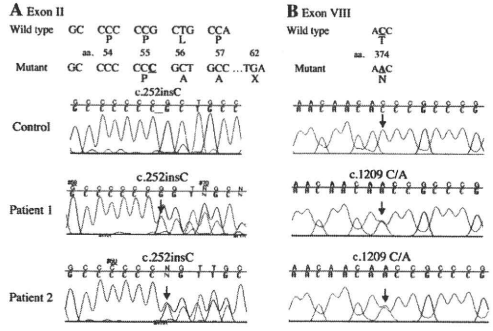

Fig. 5. Direct sequencing of *IDUA* showed 1 bp insertions, c.252insC (p.P55fs62X) (arrows), in exon II (A), and point mutations, c.1209C to A (p.T374N) (arrows), in exon VIII (B) in Patients 1 and 2.

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mild joint contracture. It is remarkable that cervical myelopathy due to pachymeningeal hypertrophy is the opportunity of the diagnosis of MPS-I in our patients. Cervical pachymeningeal hypertrophy has been reported during the clinical course in some cases of MPS-I [4,5]; however, there have been no reports of cervical myelopathy as an initial and cardinal symptom of MPS-I. Meningeal hypertrophy or hypertrophic pachymeningitis may occur in association with several underlying disease such as infection, autoimmune inflammatory disorders or malignancies [12]. MPS-I should be included in the differential diagnosis of pachymeningeal hypertrophy even if other features of MPS-I are absent or trivial.

Over a hundred *IDUA* mutations have been reported in MPS-I; some mutations may be associated with the severity of the disease [2]. Generally, insertion mutations cause frameshift preventing production of the functional enzyme, whereas missense mutations may allow some residual enzymatic activity [1]. In our patients with mutations of c.252insC (p.P55fs62X) and c.1209C>A (p.T374N), it is conceivable that the product of the allele with the c.1209C>A mutation may allow some enzymatic activity, contributing to the attenuated phenotype of MPS-I in our patients.

Our two patients demonstrated quite similar clinical manifestations. Several common mutations of *IDUA* have been reported to be mild or severe, such that the presence of two severe mutations in a patient predicts a severe phenotype, while the presence of at least one mild mutation in a patient predicts an attenuated phenotype [2]. MPS-I patients who have the identical genotype would generally show the same disease severity, for example, homozygosity or compound heterozygosity for the severe mutations, W402X and Q70X (i.e. W402X/W402X, Q70X/Q70X, and W402X/Q70), predicts a severe phenotype, whereas patients with at least one mild R89Q mutation are predicted to have an attenuated phenotype [2]. However, some mutations are known to show the variable severity of the disease even if the patients had identical mutations of *IDUA* [2]. Interestingly, patients with homozygote of P533R missense mutation were reported to be associated with various forms of the disease including both the attenuated [13] and severe [14] forms, suggesting that the disease phenotypes would not be determined only by the genotypes of *IDUA*, but would also be influenced by other genetic or environmental factors [15]. Our patients were monozygotic twins with a quite similar, unique phenotype, suggesting that genetic factors not only *IDUA* mutation but also genes other than *IDUA* may play major roles in determining the phenotypes of MPS-I. Further case studies are needed to clarify the factors that influence the phenotype of MPS-I.

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4．ライソゾーム病の診断

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Summary　**ライソゾーム病の診断のプロセスについて，おもにムコ多糖症を中心に説明した。ライソゾーム病の診断は，「蓄積物質の同定」，「ライソゾーム酵素の活性測定」，「遺伝子変異の同定」を有機的に組み合わせて行われる。酵素補充療法などの治療法が開発されたことから早期診断の意義が高まり，新生児マススクリーニングへの導入も検討されている。**

はじめに

ライソゾーム病は，ライソゾーム酵素の先天的欠損によりライソゾーム内に分解できない脂質や糖質が過剰蓄積するために，複数の臓器の障害が進行性に現れる単一遺伝子病である。診断のはじめのステップは，特徴的な臨床症状や経過からライソゾーム病を疑うことである。特定の疾患あるいは疾患群が疑えると判断された場合に確定診断へと進む。確定診断には「蓄積物質の同定」，「ライソゾーム酵素の活性測定」，「遺伝子変異の同定」が用いられる。本稿では，比較的頻度が高く系統的な診断プロセスが確立しているムコ多糖症について詳しく述べる。

1．ムコ多糖症について

ムコ多糖は体の主要な構成成分のひとつである。ムコ多糖はライソゾームに局在する約10種類の酵素によって段階的に分解される[1]。酵素のひとつが欠損していると分解が途中でとまり，高分子化合物がライソゾーム内に過剰蓄積し，細胞障害の原因となる。ムコ多糖症は臨床症状と欠損酵素の違いにより，7つの病型に分類される（表1）。遺伝形式については，ムコ多糖症Ⅱ型がX連鎖劣性遺伝病で原則として男児にのみ発症するが，その他の疾患は常染色体劣性遺伝病であり，罹患者の性差はない。

2．尿中ムコ多糖の分析

主なムコ多糖には，デルマタン硫酸（DS），ヘパラン硫酸（HS），ケラタン硫酸（KS）などがある。ムコ多糖症では尿中にムコ多糖が過剰排泄される。ムコ多糖を分解する酵素は，上記の3つの物質の1種類あるいは2種類の分解に関与する。たとえば，ムコ多糖症Ⅰ型の欠損酵素であるα-L-イズロニダーゼとムコ多糖症Ⅱ型の欠損酵素L-イズロネートスルファターゼは，DSとHSの両方の分解に必要であるので，この2疾患ではDSとHSが同時に蓄積することになる。一方，ムコ多糖症Ⅵ型の欠損酵素であるアリルスルファターゼBは，DSの分解には関与するが，HSの分解には関与しないので，DSの蓄積はあるがHSは蓄積しない。このように，尿中のムコ多糖の蓄積パターンを調べることにより，ある程度の病型予測ができる。

3．酵素活性測定

尿中ムコ多糖分析から病型がある程度絞り込まれた場合，特定の疾患の酵素活性を測定することにより診断を確定できる。検体としては，血液中の白血球を用いる。酵素活性測定には，人工基質を用いて，4メチルウンベリフェロンの蛍光発色の強度を定量することが一般的である。

表1　ムコ多糖症の病型分類

病型（略号）		欠損酵素	蓄積物質・尿中異常ムコ多糖	遺伝形式
Hurler	(MPS Ⅰ H)	α-L-Iduronidase	デルマタン硫酸 ヘパラン硫酸	常染色体性劣性遺伝
Hurler/Scheie	(MPS Ⅰ H/S)			
Scheie	(MPS Ⅰ S)			
Hunter, severe	(MPS Ⅱ, severe or attenuated)	Iduronate sulfatase	デルマタン硫酸 ヘパラン硫酸	X連鎖性劣性遺伝
Hunter, intermediate	(MPS Ⅱ, intermediate)			
Hunter, mild	(MPS Ⅱ, mild)			
Sanfilippo A	(MPS Ⅲ A)	Heparan N-sulfatase	ヘパラン硫酸	常染色体性劣性遺伝
Sanfilippo B	(MPS Ⅲ B)	α-N-Acetylglucosaminidase		
Sanfilippo C	(MPS Ⅲ C)	Acetyl CoA：α-glucosaminide acetyltransferase		
Sanfilippo D	(MPS Ⅲ D)	N-Acetylglucosamine 6-sulfatase		
Morquio A	(MPS Ⅳ A)	Galactose 6-sulfatase	ケラタン硫酸	常染色体性劣性遺伝
Morquio B	(MPS Ⅳ B)	β-Galactosidase		
Maroteaux-Lamy	(MPS Ⅵ)	N-Acetylgalactosamine 4-sulfatase (arylsulfatae B)	デルマタン硫酸	常染色体性劣性遺伝
Sly	(MPS Ⅶ)	β-Glucuronidase	デルマタン硫酸 ヘパラン硫酸 コンドロイチン硫酸A，C	常染色体性劣性遺伝

ムコ多糖症の病型別の欠損酵素，蓄積物質，遺伝形式を示す。病型により，蓄積するムコ多糖の種類が異なる。
（筆者作成）

DS（デルマタン硫酸）　HS（ヘパラン硫酸）　KS（ケラタン硫酸）

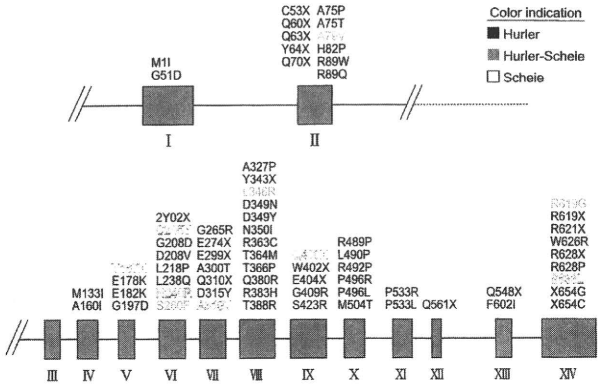

図1　ムコ多糖症Ⅰ型の遺伝子変異

図はムコ多糖症Ⅰ型のこれまでに報告された遺伝子変異のなかで，ミスセンス変異とナンセンス変異だけを示している。変異は多彩であり，ホットスポットはない。（筆者作成）

4．遺伝子解析

ムコ多糖症の原因となるライソゾーム酵素をコードする遺伝子はすべて解明されている。したがって，白血球細胞のDNAなどを用いることにより，遺伝子変異を見出し診断に結びつけることができる。遺伝子解析には以下のような問題点がある。

1）遺伝子変異のホットスポットがないので，症例ごとに遺伝子全体を解析する必要があり，時間，労力，コストがかかる。

2）遺伝子変異には，ミスセンス変異，ナンセンス変異，スプライシング異常などの比較的微細な異常から，遺伝子の組み換えや広範囲な欠失のような大きな異常まで多種多様な異常が存在する（**図1**）。

3）アミノ酸がひとつだけ置換するような変異（ミスセンス変異）の場合，すでに報告がある変異であれば疾患との関連は強いとされるが，これまでに報告されていない変異の場合，まれな多型の可能性も否定できないので注意を要する。

ムコ多糖症は，原則的には酵素活性の測定により確定診断される。遺伝子解析は診断に必須ではない。しかし，ムコ多糖症Ⅱ型の保因者診断を正確に行うためには，遺伝子解析は不可欠である。

5．出生前診断について

ムコ多糖症は難治性の疾患である。しかし，Ⅰ型，Ⅱ型，Ⅵ型では酵素補充療法が開発されているが，中枢神経症状に対する効果は期待できない。また，その他の病型，特に中枢神経症状が強く出るⅢ型については，有効な治療手段は今のところ全く存在しない。そのため，疾患患者を持つ家族では，発端者の次の子に対して出生前診断を希望する家族も少なくない。出生前診断は，絨毛細胞や羊水細胞の酵素活性を測定することで可能である。また，予め発端者の遺伝子解析の原因となる変異が確定している家族においては，絨毛細胞や羊水細胞を用いた遺伝子解析が有用である。なお，出生前診断を行う場合は，疾患の診療経験が豊富な専門医による遺伝カウンセリングを検査前に行い，検査の限界やメリット，デメリットなどを検査を受ける人に十分に理解してもらう必要がある。

6．新生児スクリーニング（NBS）について

ほとんどのライソゾーム病は，新生児期には目立った症状がない。ムコ多糖などの高分子物質が蓄積し種々の臓器障害を呈するまでには，時間がかかるからである。ムコ多糖症の場合，1～2歳で診断される場合もあるが，5～6歳まで診断されずにいることも多い。酵素補充療法や造血幹細胞移植の効果を考えると，早期にまだ目立った症状が出現する以前に治療を始めることが望ましい。たとえば，ムコ多糖症の骨病変については，症状が明らかになった後では，どのような治療をしても十分な効果は得られない。これに対して，発症前に診断を確定し治療を開始すると，骨病変などの進展に対する予防的な効果が期待できる。そのため，少なくとも治療が可能となった疾患については，新生児マススクリーニングを導入すべきという意見がある。しかし，ライソゾーム病の多くが中枢神経障害があり，現在可能な治療のいずれもが十分な治療効果がないことを考慮すると，新生児マススクリーニングの対象疾患とするには，倫理的な配慮も必要になろう[2]。

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REVIEW (Molecular Regulation of Mineralization and Its Failure)

Heterotopic Bone Formation Induced by Bone Morphogenetic Protein Signaling : Fibrodysplasia Ossificans Progressiva

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Key words : bone morphogenetic protein／genetic disease／receptor／signal transduction

Abstract : Bone morphogenetic proteins (BMPs) were originally identified *via* their unique activity in demineralized bone matrix—they induce heterotopic bone formation in skeletal muscle. BMP activity is transduced by two types of BMP-specific transmembrane serine/threonine kinase receptors and downstream transcription factors, known as Smads. Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder characterized by progressive heterotopic bone formation in skeletal muscle tissue. In patients with FOP, heterozygous mutations involving single amino-acid substitutions have been identified in the BMP type I receptor, ALK2. These mutations in ALK2 are scattered in an intracellular domain containing the kinase and GS domains. The mutant receptors constitutively activate downstream intracellular signaling, even in the absence of BMPs. In FOP, muscle injury induces acute heterotopic bone formation. Smad1 and Smad5 are up-regulated during muscle regeneration and induce osteoblastic differentiation of myoblasts in cooperation with mutant ALK2. Some types of BMP receptor inhibitors block the signal transduction induced by mutant ALK2 receptors *in vitro* and *in vivo*. Taken together, this work demonstrates that BMP signaling induces heterotopic bone formation in skeletal muscle. Inhibitors of BMP receptors may aid in the establishment of novel treatments to prevent heterotopic bone formation in FOP.

Introduction

Skeletal tissues are formed *via* two related but distinct processes during development in vertebrates : endochondral ossification and intramembranous ossification[1,2]. In endochondral ossification, undifferentiated mesenchymal cells condensed in skeletal muscle tissue are first committed to differentiate into chondrocytes. They form a cartilaginous model of bone tissue and gradually differentiate into proliferating and hypertrophic chondrocytes. The calcified cartilage formed by terminally differentiated hypertrophic chondrocytes is destroyed by chondroclasts, which derive from macrophages invading blood vessels, and is replaced by bone matrix secreted from osteoblasts. In contrast, cells undergoing mesenchymal condensation differentiate into osteoblasts and directly form bone tissue during intramembranous ossification. These developmental processes are orchestrated by various regulators, such as systemic hormones and local cytokines.

Bone morphogenetic proteins (BMPs) play critical roles in skeletal development by regulating the proliferation, differentiation, and apoptosis of chondrocytes, osteoblasts, and osteoclasts[3,4]. Although BMPs are multifunctional factors in vertebrates and invertebrates, they were originally discovered due to their bone-inducing activity in bone matrix in 1965. Urist[5] first prepared demineralized bone by treating bone matrix with hydrochloric acid and then implanted it in skeletal muscular tissues to induce re-calcification of the matrix. A few weeks after transplantation, new cartilage and bone tissues with bone marrow, rather than simple re-calcification, were ectopically formed in the demineralized bone matrix (Fig. 1). These findings suggested that the demineralized bone matrix contained unknown bioactive molecule(s) capable of inducing the differentiation of chondrocytes and osteoblasts from mesenchymal cells in muscular tissues. This ectopic bone-inducing activity was subsequently named "bone morphogenetic protein" because it was sensitive to trypsin digestion[6].

Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder characterized by congenital malformation of the big toes and progressive heterotopic bone formation in skeletal muscle tissue[7,8]. The frequency of FOP is estimated at one per 2 million, regardless of race, sex, and area of the world[8]. Genes encoding BMP signaling molecules have been suggested as possible candidates responsible for FOP because the process of heterotopic bone formation in FOP is similar to that induced by BMP implantation in muscle tissue[7]. In 2006, a concurrent heterozygous mutation in the *ACVR1* gene was identified from both familial and sporadic FOP patients[9]. Their *ACVR1* genes had a substitution mutation of guanine to adenine in position 617 ; this changes histidine to arginine in position 206 of the type I BMP receptor ALK2[9]. The mutant receptor, ALK2 (R206H), activates intracellular signaling specific to BMPs, even in the absence of ligands[10] ; therefore, it is considered a constitutively active BMP receptor. Moreover, the process of muscle regeneration seems to enhance BMP signaling by increasing downstream transcription factors[10]. Taken together, these findings shed light on the molecular mechanisms underlying pathological bone formation in skeletal muscle tissue by BMP signaling and the development of new treatments for FOP.

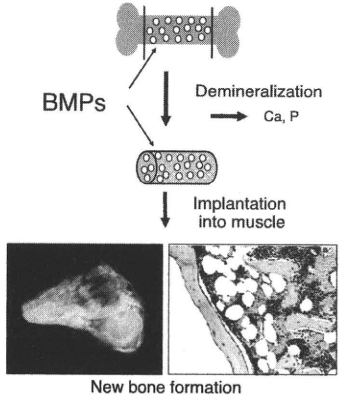

Fig. 1 Ectopic bone formation induced by BMPs
BMPs are stored in bone matrix and retained after demineralization by HCl. Implantation of the demineralized bone matrix containing BMPs into skeletal muscle induces heterotopic bone formation after 1—2 weeks.

Signal Transduction of BMPs

BMPs, except BMP-1, are members of the transforming growth factor-β (TGF-β) family[3]. Heterotopic bone-inducing activity in skeletal muscle is conserved between several BMP family members, including BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-9, and GDF-5 but not BMP-3, TGF-β, or activin[11]. The difference in bone-inducing activity *in vivo* can be reproduced in C2C12 myoblast cultures *in vitro* ; BMPs inhibit myogenesis and induce osteoblastic differentiation, but TGF-β1 fails to induce osteoblastic differentiation even though it inhibits myogenesis[12]. C2C12 myoblasts are widely used today for studying the molecular mechanisms of heterotopic bone formation by BMP signaling *in vitro*.

Similar to other members of the TGF-β family, the

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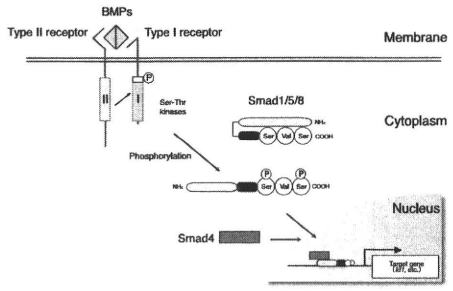

Fig. 2 Signal transduction of BMPs

BMPs bind to type I and type II receptors, which are transmembrane serine/threonine kinases expressed on the cell membrane. The type II receptor phosphorylates the type I receptor at a "GS domain", then the activated type I receptor phosphorylates Smad1/5/8 in the cytoplasm. Phospho-Smad1/5/8 form complexes with Smad4, move into the nucleus, and regulate gene expression.

intracellular signal transduction of BMPs is activated by two types of transmembrane serine/threonine kinase receptors : type I and type II receptors (Fig. 2)[3,13]. Combinations among three types of type II (BMPR-II, ActR-II, and ActR-IIB) and four types of type I (ALK1, ALK2, BMPR-IA/ALK3, and BMPR-IB/ALK6) receptors bind to BMPs and activate BMP-specific biological activities[3,13]. In contrast to TGF-β/activin receptors, BMP type I receptors do not seem to require type II receptors for ligand binding. In the ternary complex containing the BMP-type II receptor and type I receptor, the type II receptor phosphorylates the type I receptor at the "GS domain", which consists of a glycine and serine residue-rich domain located intracellularly between the transmembrane and kinase domains of type I receptors. The phosphorylated type I receptor at the GS domain activates downstream signaling pathways, including Smads, p38 MAP kinase, and PI3 kinase, *via* phosphorylation.

The GS domain of the type I receptor serves as a "molecular switch" for kinase activity. Substitution of a conserved asparagine residue in the GS domain of the type I receptors (which is present not only among BMP receptors but also TGF-β and activin receptors) activates kinase activity without stimulation by ligands or type II receptors[14]. A kinase domain-truncated type I receptor, which still retains a ligand binding domain, blocks the biological activities of BMPs as a dominant negative receptor[15,16]. These findings suggest that type I receptors play a central role in the signal transduction of BMPs due to phosphorylation-dependent regulation at the GS domain.

Among the intracellular signaling pathways activated by BMP type I receptors, the Smad-dependent pathway seems to be critical for bone formation induced by BMPs[13]. Smads are the mammalian counterparts of Sma and Mad in *C. elegans* and *Drosophila*, respectively, that transduce intracellular signaling of the TGF-β family of ligands. Smads are divided into three subgroups based on their structures and functions : receptor-regulated Smads (R-Smads), common Smads (Co-Smads), and inhibitory Smads (I-Smads)[3,13]. R-Smads are phosphorylated at the carboxyl termini by type I receptors, form ternary complexes with Co-Smad and Smad4, and move into nuclei to regulate the transcription of the target genes (Fig. 2). Specificity of ligand activity depends at least in part on receptor-activated Smads ; BMP receptors mainly phosphorylate Smad1, Smad5 and Smad8, but TGF-β/activin receptors phosphorylate Smad2 and Smad3.

When activated by BMP type I receptors, Smad1 is introduced negative charges at both the Ser463 and Ser465 positions *via* phosphorylation. Substitution of these serine residues by aspartic acid, which is an acidic amino acid with an additional negative charge, seems to reproduce a state similar to phosphorylation[17]. This mutant Smad1, called Smad1 (2SD) or Smad1(DVD), is a constitutively activated Smad1 because it activates target gene expression (such as that of *Id1*) and induces osteoblastic differentiation of C2C12 myoblasts in cooperation with Smad4 in the absence of ligands or type II or type I receptors[17] (unpublished observation). A specific chemical inhibitor of BMP-Smad phosphorylation, but not MAP kinase inhibitors, inhibits the osteoblastic differentiation of C2C12 cells induced by BMPs. Taken together, these findings suggest that the BMP receptor-Smad axis plays an important role in heterotopic bone formation induced by BMPs.

Clinical Features of FOP

FOP is an autosomal dominant disorder characterized by congenital malformation of the big toes and progressive heterotopic bone formation in skeletal muscle tissues that causes joint fusion with resultant permanent immobility[8]. The diaphragm, tongue, extra-ocular muscles, cardiac muscle and smooth muscles are not involved in the bone formation in FOP[8]. Almost all patients with FOP have malformation of the big toes at birth, suggesting that a protein encoded by the gene responsible for FOP regulates the patterning of the big toes during development[7,8]. A conductive hearing impairment is commonly observed in patients with FOP.

Prior to heterotopic bone formation in skeletal muscle, FOP patients develop painful flare-ups composed of inflammatory soft tissue swellings that contain a large number of mast cells[8]. The heterotopic bones are usually formed at flare-up sites *via* the process of endochondral bone formation. Minor trauma, such as intramuscular immunizations, mandibular blocks for dental work, overstretching of the jaw, muscle fatigue, blunt muscle trauma from bumps, bruises, falls, or influenza-like illnesses, is known to trigger new flare-ups of FOP and lead to heterotopic bone formation. Surgical operation and biopsy are prohibited in FOP because they also lead to new flare-ups and heterotopic bone formation. Thus, the heterotopic bone formation in FOP is an event inducible by muscle injury and muscle regeneration.

In addition to typical FOP features, some additional and/or different clinical features have been identified in atypical FOP[18]. These include severe but variable reduction deficits of the digits, absent finger/toenails of digits with severe reduction deficits, sparse and/or thin scalp hair, minimal change to the big toe, and slow progression of heterotopic bone formation in muscle. Recently, it has been reported that these differences are caused by different mutations in the *ACVR1* gene (see below).

Mutations in ALK2 Identified from FOP Patients

Several proteins and genes related to BMP signaling have been suggested to be responsible for FOP. The overexpression of BMP-4 was observed in lymphocytes prepared from FOP patients. Deletion of *NOGGIN* at 17q21-22, which encodes a BMP antagonist, was identified in a FOP patient ; however, this mutation was not detected by others and was excluded by linkage analysis[19]. Additional point mutations in the *NOGGIN* gene have been reported in FOP patients[20]. Genome-wide linkage analysis identified the 4q27-31 region as responsible for FOP, and this region contains *SMAD1*[21].

In 2006, the FOP mutation was mapped to chromosome 2q23-24, which contains the *ACVR1* gene encoding the BMP type I receptor ALK2[9]. A recurrent mutation (617G>A) was identified in the *ACVR1* gene in all examined individuals with FOP, including 5 familial and 32 sporadic cases (Fig. 3). This mutation was also confirmed in a patient who has been reported to carry a *NOGGIN* mutation[9]. In addition, this mutation was also identified in at least 22 Japanese FOP patients[10,22].

This *ACVR1* 617G>A mutation causes an amino

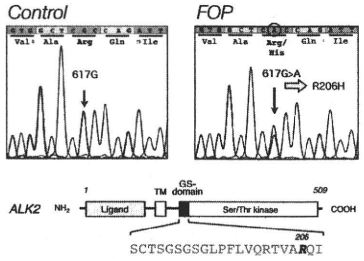

Fig. 3 R206H mutation in ALK2 identified in patients with FOP

The *ACVR1* gene encodes the BMP type I receptor ALK2. The control *ACVR1* gene has a "G" at position 617, but alleles from patients with FOP show heterozygosity for "G" and "A"; this results in a change of arginine to histidine residue at position 206 in the ALK2 protein. Arg206 is localized in the GS domain, which is a "molecular switch" for the serine/threonine kinase activity of ALK2.

acid change from arginine to histidine residues in codon 206 (Fig. 3)[9]. Arg206 is highly conserved among vertebrates and a proximal amino acid of Gln207; a substitution mutation in Gln207 to glutamic acid has been reported to activate constitutive kinase activity[14]. Indeed, overexpression of ALK2 (R206H) in C2C12 myoblasts inhibits myogenesis and induces the nuclear accumulation of phospho-Smad1/5/8, *Id1* promoter/enhancer activity, and alkaline phosphatase activity[10]. Up-regulation of endogenous *ID1* expression and activation of p38 MAP kinase are also observed in lymphocytes immortalized from patients with FOP[23]. These findings clearly indicate that ALK2(R206H) is a genetically activated BMP type I receptor. This is the first case of a gain-of-function mutation among BMP type I receptors.

In addition to ALK2(R206H), several mutations in the same *ACVR1* gene at different positions have been identified in patients with atypical FOP. These novel mutations are scattered between the GS domain and kinase domain, and include delP197-F198 insL, R202I, Q207E, R258S, G328E, G328R, G328W, G356D, and R375P[18,24–26]. The structural changes of mutant ALK2 (including R206H) have been analyzed in silico, and this analysis suggests that they exhibit reduced ability to bind to FKBP12 (a binding repressor of the BMP/TGF-β type I receptors)[27]. It has also been suggested that a substitution of Arg206 to histidine creates a pH-sensitive switch in the GS domain that leads to ligand-independent activation of ALK2[27].

Muscle Regeneration and Ectopic Bone Formation

In FOP patients, muscle injury induces acute heterotopic bone formation, suggesting that the process of muscle regeneration may stimulate BMP signaling. A snake venom injection into skeletal muscle tissue in mice induces the whole process of muscle regeneration within a couple of weeks. In this model, expression of Smad1 and Smad5, but not Smad8, is up-regulated within 3 days of injection[10]. Smad1 and Smad5 are expressed in mononuclear cells appearing in the regenerating muscle. Although both Smad1 and Smad5 are not phosphorylated in wild-type mice *in vivo*, they become phosphorylated and activated for osteoblastic differentiation after co-transfection with ALK2(R206H) in C2C12 myoblasts[10]. Thus, cooperation of genetically activated mutant ALK2 and increased Smad1 and Smad5 may induce downstream signaling to induce heterotopic bone formation in skeletal muscle in FOP (Fig. 4). Injection of snake venom into skeletal muscle also stimulates heterotopic bone formation in BMP-4-expressing transgenic mice.

Clinical features of FOP suggest that myogenic cells are potential progenitors of the chondrocytes and osteoblasts forming heterotopic skeletal tissue in patients with FOP. In addition, the activity of ALK2 (R206H) in C2C12 cells further confirms this hypothesis[10]. These cells are believed to be derived from satellite cells, which are immature progenitor cells of myoblasts in skeletal muscle tissue. Recently, Tie2-expressing cells were identified as progenitor cells that contribute heterotopic cartilage and bone

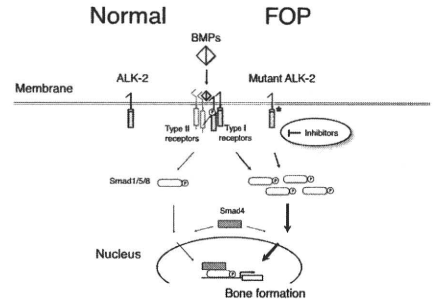

Fig. 4 A schematic model of signal transduction in FOP

Under normal conditions, ALK2 induces bone formation *via* Smad1/5/8 only after binding to BMP ligands. In contrast, in FOP, mutant ALK2 phosphorylates Smad1/5/8 and induces signal transduction without binding to BMPs. Moreover, levels of expression of Smad1 and Smad5 increase during muscle regeneration and mediate the mutant ALK2-stimulated bone formation. Inhibitors of ALK2, such as Dorsomorphin and LDN-193189, may aid in the establishment of novel treatments for FOP.

formation induced by BMP-2 using a Cre/LoxP tracing system *in vivo*[28]; however, MyoD-expressing cells did not differentiate into either chondrocytes or osteoblasts in this model. These findings suggest that endothelial progenitors, but not myoblasts, contribute to heterotopic bone formation in FOP.

Establishment of New Treatments for FOP

Today, there is no effective treatment for preventing heterotopic bone formation in FOP; however, the establishment of new treatments for FOP has begun based on the recent findings regarding ALK2. Since the genetic activation of ALK2 activity seems to play a central role in heterotopic bone formation in FOP, specific inhibitors of the BMP receptors have been developed to block intracellular signaling from mutant ALK2. Dorsomorphin is a specific small chemical inhibitor of BMP type I receptors, such as ALK2, BMPR-IA and BMPR-IB[29]. Interestingly, Dorsomorphin inhibits the phosphorylation of Smads, but not p38, induced by BMPs[29]. Treatment of C2C12 cells expressing ALK2(R206H) or ALK2(G356D) with Dorsomorphin blocks the induction of osteoblastic differentiation *in vitro*[10]. In addition, overexpression of Smad7 also blocks the ALK2(R206H)-induced osteoblastic differentiation of C2C12 cells. Smad6 shows only minimal activity in this model.

LDN-193189 is a potent derivative of Dorsomorphin and inhibits the BMP-4-induced phosphorylation of Smad1/5/8 at an approximately 80-fold lower concentration than Dorsomorphin *in vitro*[30]. Yu *et al.*[30] established a new *in vivo* model system of heterotopic bone formation in skeletal muscle using mice carrying a floxed ALK2(Q207D) mutation; however, it should be noted that this mutation has not been found in patients with FOP. An injection of Cre-expressing adenovirus into skeletal muscle of floxed ALK2(Q207D) mice induces local heterotopic bone formation, joint fusion and functional impairment similar to that in FOP. Treatment of these mice with LDN-19318 reduces heterotopic bone formation and functional impairment, suggesting that this type of inhibitor may be useful for curing FOP (Fig. 4)[30].

In the early stages of FOP research, it was suggested that bone marrow transplantation would be

effective in preventing heterotopic bone formation in FOP since increased BMP-4 expression in lymphocytes seemed to be responsible for this disorder[31]. In 2007, a report described a FOP patient with the ALK2 (R206H) mutation who had received bone marrow transplantation at age 10 and thereby avoided heterotopic bone formation for 14 years while undergoing immunosuppressive treatments[32]. However, the patient experienced heterotopic bone formation after cessation of the immunosuppressive treatments, suggesting that the immune system may also contribute to heterotopic bone formation in FOP[32].

Conclusion

BMP signaling is a potent inducer of heterotopic bone formation in skeletal muscle tissue. Activation of the type I receptor-Smad axis plays a central role in the bone formation induced by BMPs. Patients with FOP have mutations in ALK2 that exist as constitutively activated forms of the BMP type I receptor. Muscle regeneration in FOP may cooperatively stimulate heterotopic bone formation by increasing Smad1/5 expression. Development of specific inhibitors for ALK2 would be useful to prevent heterotopic bone formation in FOP.

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Dual Roles of Smad Proteins in the Conversion from Myoblasts to Osteoblastic Cells by Bone Morphogenetic Proteins*[S]

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Bone morphogenetic proteins (BMPs) induce ectopic bone formation in muscle tissue *in vivo* and convert myoblasts such that they differentiate into osteoblastic cells *in vitro*. We report here that constitutively active Smad1 induced osteoblastic differentiation of C2C12 myoblasts in cooperation with Smad4 or Runx2. In floxed Smad4 mice-derived cells, Smad4 ablation partially suppressed BMP-4-induced osteoblast differentiation. In contrast, the BMP-4-induced inhibition of myogenesis was lost by Smad4 ablation and restored by Smad4 overexpression. A nuclear zinc finger protein, E4F1, was identified as a possible component of the Smad4 complex that suppresses myogenic differentiation in response to BMP signaling. In the presence of Smad4, E4F1 stimulated the expression of *Ids*. Taken together, these findings suggest that the Smad signaling pathway may play a dual role in the BMP-induced conversion of myoblasts to osteoblastic cells.

Bone morphogenetic proteins (BMPs)[2] are members of the transforming growth factor-β (TGF-β) superfamily, which regulates the differentiation, proliferation, and death of various types of cells (1). BMPs were originally found in bone matrix as factors responsible for the induction of ectopic bone formation, in which implantation of demineralized bone matrix into muscle tissue induced new bone tissue containing bone marrow (2, 3). Implantation of individual recombinant BMPs, such as BMP-2, BMP-4, BMP-6, and BMP-7, into muscle tissue induces ectopic bone formation *in vivo* as well (4). This ectopic bone-inducing activity is highly specific to BMPs, because other hormones and cytokines, including TGF-β1 itself, failed to induce ectopic bone formation in muscle tissue *in vivo* (5). Although many factors, such as BMPs, TGF-βs, fibroblast growth factors, and epidermal growth factor, inhibit myogenic maturation of myoblasts *in vitro*, only BMPs convert them so that they differentiate to osteoblastic cells, the bone-forming cells in vertebrates (6–10). Thus, the activity of BMPs in myoblast cultures to induce osteoblastic differentiation appears to reflect the ectopic bone-inducing activity of BMPs *in vivo* (8).

BMP signaling is transduced by two different types of serine/threonine kinase receptors, termed type I and II receptors (1, 11, 12). The BMP-bound type II receptor phosphorylates the type I receptor kinase, and the activated BMP type I receptor in turn phosphorylates downstream substrates such as receptor-regulated Smads (R-Smads), including Smad1, Smad5, and Smad8 and p38 mitogen-activated protein kinase. Phosphorylated R-Smads form heteromeric complexes with Smad4 and translocate into the nucleus to regulate transcription of various target genes, including *Id1*, which encodes a dominant-negative inhibitor of myogenesis (13–15). Recently, a genetic mutation of ALK2, a BMP type I receptor, was identified in patients with fibrodysplasia ossificans progressiva, an autosomal-dominant disorder characterized by heterotopic bone formation in muscle tissue (16). Our findings indicate that this mutant ALK2 is a constitutively activated and hyper-reactive form of the BMP type I receptor and suggest that downstream signaling of activated receptors play an important role in heterotopic bone formation in muscle tissue under certain pathological conditions (17).

Here, we show that a protein in which the carboxyl-terminal serine residues of Smad1 were substituted with aspartic acids, termed Smad1(DVD), functioned as a constitutively activated Smad1. Overexpression of Smad1(DVD) induced osteoblastic differentiation of C2C12 myoblasts, although the inhibition of myogenic differentiation depended principally on nuclear Smad4 rather than R-Smads. E4F1 was identified as a possible component of the Smad4 complex in the suppression of myogenic differentiation by BMP signaling. R-Smads and Smad4 play important roles in the conversion of myogenic cells to osteoblastic cells by BMPs, data were confirmed with the deletion of Smad4 in mouse embryonic fibroblasts (MEFs).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[2] The abbreviations used are: BMP, bone morphogenetic protein; ALP, alkaline phosphatase; BMPR-IA, bone morphogenetic protein receptor type IA; MHC, myosin heavy chain; TGF-β, transforming growth factor-β; ChIP, chromatin immunoprecipitation; NLS, nuclear localization signal; EGFP, enhanced green fluorescent protein; MEF, mouse embryonic fibroblast; RNAi, RNA interference; siRNA, small interfering RNA; R-Smad, receptor-regulated Smad; luc, luciferase.

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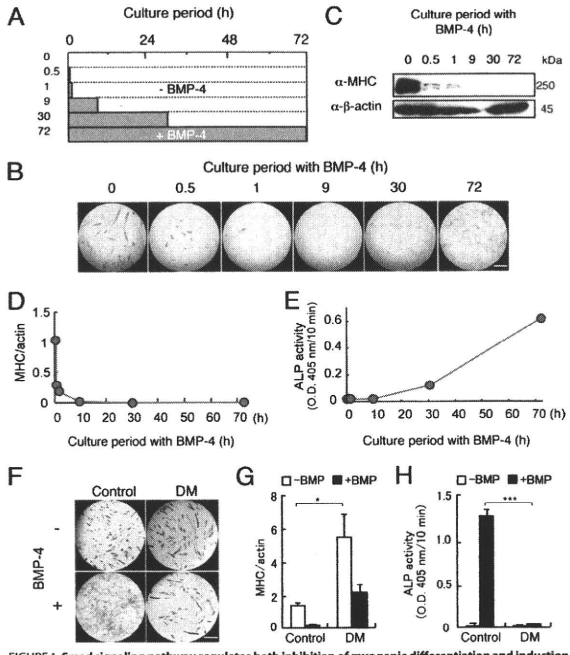
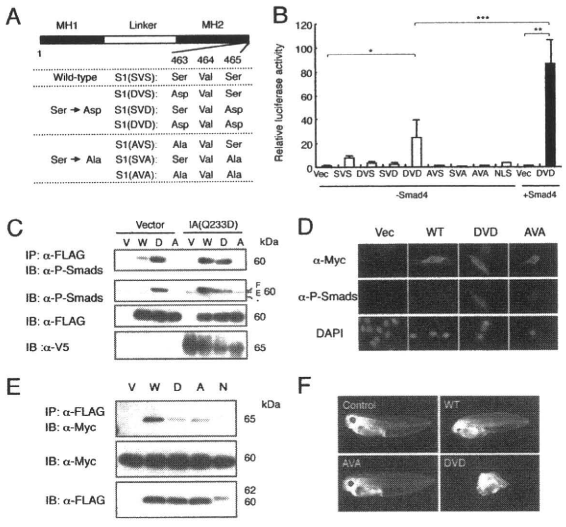


FIGURE 1. **Smad signaling pathway regulates both inhibition of myogenic differentiation and induction of osteoblastic differentiation by BMP-4.** *A* and *B*, examination of the minimal treatment periods required for inhibition of myogenic differentiation and induction of osteoblastic differentiation by BMP-4 in C2C12 myoblasts. *A*, scheme of treatment of C2C12 myoblasts with BMP-4 in a window experiment. C2C12 cells were treated for 0, 0.5, 1, 9, 30, or 72 h with 100 ng/ml of BMP-4 (*closed bars*) and further incubated without BMP-4 (*open bars*) until 72 h before staining. *B*, the cells were doubly stained for ALP and MHC in *blue* and *red*, respectively, at 72 h. *Scale bar*, 200 μm. *C–E*, quantitation of the effects of BMP-4 on myogenic differentiation and osteoblastic differentiation of C2C12 cells. Western blots for MHC (*C* and *D*) and measurement of ALP activity (*E*) were performed on day 3. *F–H*, effects of Dorsomorphin on BMP-induced differentiation in C2C12 cells. C2C12 cells were preincubated for 1 h with 3 μM Dorsomorphin (DM) and 100 ng/ml of BMP-4 was then added. *F*, the cells were doubly stained for ALP and MHC on day 3. *Scale bar*, 400 μm. MHC levels (*G*) and ALP activity (*H*) were measured on day 3. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$.

EXPERIMENTAL PROCEDURES

Expression Vectors—Expression vectors for wild-type mouse Smad1, Smad4, and Smad7 were described previously (13). All of the mutations were introduced by a standard PCR technique using Platinum Pfx DNA polymerase (Invitrogen) and their sequences were confirmed in each expression vector. Smad1 mutants were obtained by substitution of Ser-463 and/or Ser-465 of mouse Smad1 with aspartic acid or alanine residues. A nuclear localization signal derived from an SV40 large T antigen, PPKKKRKV, was inserted between an amino-terminal epitope tag and Smad1 or Smad4. NLS-Smad4(ΔMH1) and NLS-Smad4(ΔMH2) were obtained by deleting amino acids 2–138 and 316–552, respectively, from full-length NLS-Smad4. Both mouse wild-type and dominant-negative Runx2 vectors were kindly provided by Dr. Toshihisa Komori (Nagasaki University) (18). Mouse *E4f1* (accession number BC057011) cDNA was obtained by a standard reverse transcription-PCR technique and cloned into pcDEF3 with a FLAG epitope sequence at the 3′ end (19), and E4F1(ΔE3) and the zinc finger mutants (Z1–Z6) were generated by deleting a ubiquitin E3 ligase domain, amino acids 40–84, and substituting two cysteine residues with alanines at positions 195 and 197, 223 and 226, 435 and 438, 519 and 522, and 547 and 550, respectively.

Cell Cultures and Transfections—C2C12 mouse myoblasts, C3H10T1/2 mouse fibroblasts, and COS-7 African green monkey kidney cells were maintained as described (8, 20). The cells were treated with 100 ng/ml of recombinant human BMP-4 (R & D Systems, Inc.) in the presence or absence of chemical inhibitors of mitogen-activated protein kinase, U0126, SB203580, or SP600125 (Merck, Tokyo, Japan) or of the BMP Smad pathway, Dorsomorphin (Calbiochem, San Diego, CA) (20, 21). Lipofectamine 2000 (Invitrogen) was used for plasmid transfections. RNAi Stealth oligonucleotides against murine *Smad1* (MSS275578), *Smad5* (MSS2755978), *Runx2* (MSS202675), *Smad4* (MSS206437), and *E4f1* (MSS274050) and a scrambled negative control oligonucleotide were purchased from Invitrogen. An expression plasmid for *E4f1* microRNA was constructed by subcloning Mmi508063 (Invitrogen) into pcDNA6.2-GW/EmGFP-miR (Invitrogen). MEFs prepared from *Smad4*$^{flox/flox}$ mouse embryos at 11.5 days postcoitum were infected with an



	Viability	DAI (dorso-anterior index)					Average	Others
		5	4	3	2	1		
Control	34 (100)	33 (97.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5.00	1 (2.9)
Smad1 WT	34 (100)	1 (2.9)	22 (64.7)	7 (20.6)	3 (8.8)	0 (0.0)	3.64	1 (2.9)
Smad1(AVA)	34 (100)	29 (85.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5.00	5 (14.7)
Smad1(DVD)	34 (100)	0 (0.0)	0 (0.0)	9 (26.5)	9 (26.5)	16 (47.1)	1.79	0 (0.0)

FIGURE 2. **Establishment of a constitutively activated Smad1.** *A*, construction of mutant Smad1. Serine 463 and/or 465 at the carboxyl terminus of mouse Smad1 was substituted by aspartic acid or alanine. *B*, transcriptional activities of Smad1 mutants in luciferase assay in C2C12 cells. Wild-type and mutant *Smad1* were transfected with IdWT4F-luc in C2C12 cells in the presence or absence of *Smad4* ($n = 3$). Note that Smad1(DVD) activated the reporter activity even in the absence of *Smad4*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C* and *D*, Smad1(DVD) was recognized by the α-phospho-Smad1/5/8 antibody. C2C12 cells were co-transfected with empty vector (*Vector*) or V5-tagged *Bmpr-Ia*(*Q233D*) (*IA*(*Q233D*)) and an empty vector (*V*), FLAG-tagged wild-type *Smad1* (*W*), *Smad1*(*DVD*) (*D*), or *Smad1*(*AVA*) (*A*). Whole cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody followed by immunoblotting (*IB*) using α-P-Smads antibody to detect the mutant Smad1 and endogenous Smad1/5/8. *D*, C2C12 cells transfected with Myc-tagged wild-type or *Smad1*(*DVD*), *Smad1*(*AVA*), or *NLS-Smad1* were stained with α-P-Smads and α-Myc antibodies without BMP stimulation. Note that Smad1(DVD) was detected in the cytoplasm with α-P-Smads antibody. *E*, interaction with Smad1 and Smad4. C2C12 cells were co-transfected with Myc-tagged *Smad4* and an empty vector (*V*), FLAG-tagged wild-type *Smad1* (*W*), *Smad1*(*DVD*) (*D*), *Smad1*(*AVA*) (*A*), or *NLS-Smad1* (*N*). Whole cell lysates were immunoprecipitated with α-FLAG antibody followed by immunoblotting using α-Myc antibody to detect Smad4 in complex with Smad1. *F* and *G*, ventralization inducing activity of Smad1 in *Xenopus* embryos. *F*, *Xenopus* embryos at four-cell stage were injected with 500 pg of synthetic mRNA of wild-type, *Smad1*(*AVA*), or *Smad1*(*DVD*) into the dorsal marginal region. *G*, dorsal-anterior index of *Xenopus* embryos induced by Smad1. Values smaller than 5 indicate degree of ventralization.

adenovirus expressing Cre recombinase (pAxCANCre) or a human histone 2B-GFP unit (pAxH2BGFP) under control of the CAG promoter (22, 23). After being cultured for an additional 48 h, MEFs were transfected with plasmids using Lipofectamine 2000 (Invitrogen) in combinations as described in each figure legend. The cells were further cultured with or without BMP-4 before being examined for BMP activities as described below. A cell line, clone 16, was established by a limiting dilution method from *Smad4*$^{floxed/floxed}$ MEFs maintained by more than 30-fold serial passage. Bone marrow stromal cells were prepared from tibiae of 8-week-old *Smad4*$^{floxed/floxed}$ mice.

Immunohistochemistry, Immunoprecipitation, and Western Blot Analysis—The following antibodies were used for immunohistochemistry, Western blot analysis, and immunoprecipitation: anti-MHC antibody (clone MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA), anti-myogenin (clone F5D, Santa Cruz, Santa Cruz, CA), anti-FLAG antibody (clone M2, Sigma), anti-Myc antibody (clone 9E10, Santa Cruz), anti-Myc polyclonal antibody (Medical & Biological Laboratories Co., Nagoya, Japan), anti-Smad4 antibody (clone B-8, SC-7966, Santa Cruz), anti-V5 antibody (P/N 46–0705, Invitrogen), anti-E4F1 antibody (Bethyl Laboratories, Montgomery, TX), anti-phospho-Smad1/5/8 polyclonal antibody (Cell Signaling, Beverly, MA), anti-Runx2/Cbfa-1 (Medical & Biological Laboratories Co.), and anti-β-actin antibody (I-19, SC-1616, Santa Cruz). For immunohistochemical analysis, target proteins were visualized using a Histofine SimpleStain Kit (Nichirei, Tokyo, Japan) or an Alexa 488- or Alexa 594-conjugated secondary antibody (Invitrogen). A BZ-9000 (Keyence, Tokyo, Japan) microscope was used for fluorescent analysis. Western blot analysis was performed as described (17). The target proteins were immunoprecipitated for 6 h at 4 °C using M2-agarose beads (Sigma). The target proteins were detected using a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (GE Healthcare).

ALP and Luciferase Assays—ALP activity was stained as a typical marker of osteoblastic differentiation (24). Enzyme activity was measured using *p*-nitrophenyl phosphate as a substrate (24). Luciferase assays were performed using pGL3MG-185 (25) or IdWT4F-luc reporter plasmids and phRL-SV40 (Promega, Madison, WI) with the Dual-Glo Luciferase Assay System (Promega) as described previously (13).

Dorsoventral Assay in Xenopus Embryos—A dorsoventral assay in *Xenopus* embryos was performed essentially as described (26). The injected embryos were allowed to develop until stages 34–40 for observation of external appearance and then subjected to histological analysis. The activity of each Smad1 was expressed by a dorso-anterior index (26, 27).

Reverse Transcription-PCR Analysis—Total RNAs were extracted using TRIzol (Invitrogen) and reverse transcribed with SuperScript III (Invitrogen). The PCR was performed using Platinum Pfx DNA polymerase (Invitrogen) as described (28). The primer sets used were previously described (29).

Chromatin Immunoprecipitation Assay—Cells were lysed in ChIP buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and sonicated. The chromatin solution was subjected to immunoprecipitation using a OneDay ChIP Kit (Diagenode, Sparta, NJ) according to the manufacturer's instructions. The following antibodies were used: anti-E4F1 (Bethyl), anti-Smad4 (sc-7966, Santa Cruz Biotechnology), anti-MyoD (sc-760, Santa Cruz Biotechnology), and anti-histone H3 (Upstate, Lake Placid, NY). The *Myogenin* promoter was amplified by PCR using the following primers: 5′-TAATTGAAAGGAGCAGATGAGACGGGG-3′ and 5′-CCATCAGGTCGGAAAAGGCTTGTTC-3′.

Statistical Analysis—Comparisons were made using an unpaired Student's *t* test. Results are represented as mean ± S.D. Statistical significance is displayed as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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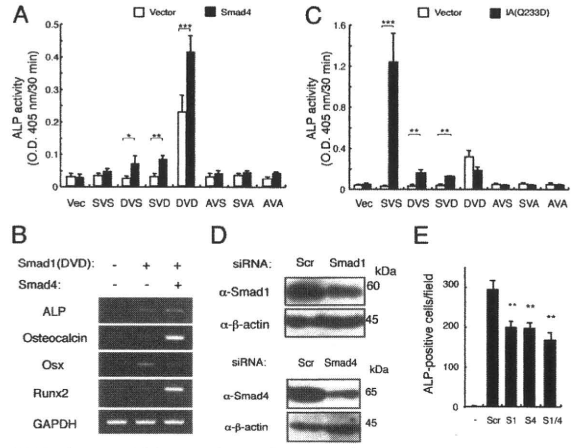

FIGURE 3. **Smad1(DVD) induces osteoblastic differentiation of C2C12 myoblasts.** *A*, Smad1(DVD) (*DVD*) induces ALP activity in C2C12 myoblasts. C2C12 cells were transfected with one of the *Smad1* constructs in the presence (*closed bars*) or absence (*open bars*) of *Smad4*, and ALP activity was measured on day 3. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, Smad4 enhances Smad1(DVD) activity. C2C12 cells were transfected with empty vector alone or with *Smad1*(*DVD*) in the absence or presence of *Smad4*. Reverse transcription-PCR was performed on day 3. *C*, synergism between Smad1 and a constitutively activated BMPR-IA receptor. C2C12 cells were transfected with one of the *Smad1* constructs in the absence (*open bars*) or presence of *Bmpr-Ia*(*Q233D*) (*closed bars*), and ALP activity was determined on day 3. Values are mean ± S.D. ($n = 3$). *D*, knockdown of Smad1 and Smad4 in C2C12 cells. C2C12 cells were transfected with 20 nM *Smad1* (*upper panels*) and *Smad4* (*lower panels*) siRNA. Protein levels were determined by Western blots at 24 h. *E*, knockdown of Smad1 or Smad4 reduced ALP activity induced by BMP-4. The siRNA-transfected C2C12 cells were treated for 3 days with 100 ng/ml of BMP-4 and ALP activity was determined. Values are mean ± S.D. ($n = 3$). **, $p < 0.01$ compared with a scrambled siRNA transfection group.

RESULTS

Induction of the Smad-dependent Pathway by BMP-4 Regulates Both Myogenic and Osteoblastic Differentiation of C2C12 Cells—First, we determined the minimal culture periods of C2C12 cells with BMP-4 required for the inhibition of myogenic differentiation and induction of osteoblastic differentiation (Fig. 1*A*). Treatment of C2C12 cells with 100 ng/ml of BMP-4 for the first 30 min of a 3-day culture markedly suppressed myogenic differentiation on day 3 but did not induce ALP activity (Fig. 1, *B–E*). ALP-positive cells were detected in cultures treated with BMP-4 for longer than 9 h (Fig. 1, *B* and *E*). The BMP-4-induced inhibition of myogenic differentiation and induction of osteoblastic differentiation were blocked by Dorsomorphin, a BMP-Smad specific inhibitor, but not by inhibitors of mitogen-activated protein kinases, suggesting that the Smad-dependent pathway regulates conversion to differentiation (Fig. 1, *F–H*, and supplemental Fig. S1).

Construction of Constitutively Activated Smad1—We generated a series of Smad1 mutants, in which one or two serine residues at the carboxyl termini were substituted with aspartic acid or alanine residues (Fig. 2*A*). Among these Smad1 mutants, Smad1(DVD) exhibited transcriptional activity in a luciferase assay using the ID1 reporter without the addition of BMPs, and this activity was further enhanced by co-transfection with Smad4 (Fig. 2*B*). NLS-Smad1, in which a nuclear localization signal (NLS) was added to the amino terminus of Smad1, failed to induce luciferase activity. Smad1(DVD) was recognized by the α-phospho-Smad1/5/8 antibody, even in the absence of a constitutively active BMP receptor, BMPR-IA(Q233D), without affecting endogenous phospho-Smad1/5/8 levels (Fig. 2, *C* and *D*). Smad1(DVD) did not exhibit changes in cellular localization or interaction with Smad4 that were distinguishable from wild-type or other Smad1 mutants (Fig. 2, *D* and *E*). Injection of synthetic *Smad1*(*DVD*) mRNA into the dorsal sides

of *Xenopus* embryos induced ventralization, although *Smad1(AVA)* did not exhibit this activity (Fig. 2*F*). The average dorso-anterior index values induced by *Smad1(DVD)* and *Smad1(AVA)* were 1.79 and 5.00, respectively, indicating that Smad1(DVD) is a constitutively activated Smad1 in *Xenopus* embryos as well (Fig. 2*G*).

Activated Smad1 and Runx2, but Not Smad4, Cooperatively Induce Osteoblastic Differentiation of C2C12 Cells—Transient transfection of *Smad1(DVD)* in C2C12 cells induced expression of osteoblastic differentiation markers such as *ALP*, *Osteocalcin*, *Runx2*, and *Osterix*; this was further enhanced by the presence of *Smad4* for every marker except *Osterix*, which might be peaked within 3 days before sample preparation (Fig. 3, *A* and *B*). BMPR-IA(Q233D) stimulated ALP activity in cooperation with wild-type Smad1, confirming that Smad1 is a critical substrate of the type I receptor for induction of osteoblastic differentiation (Fig. 3*C*). However, no synergism was observed between BMPR-IA(Q233D) and any Smad1 mutants, including Smad1(DVD), suggesting that Smad1 mutants are not recognized as substrates by the receptor (Figs. 2*C* and 3*C*). Transfection of siRNA against *Smad1*, *Smad4*, or a combination of the two reduced the ALP activity induced by BMP-4 or BMPR-IA(Q233D) (Fig. 3, *D* and *E*). Similar results were obtained using siRNA against *Smad5* (data not shown).

The role of Smad proteins in osteoblastic differentiation was further examined using MEFs prepared from *Smad4*$^{floxed/floxed}$ mice. The MEFs had been infected with an adenovirus expressing Cre recombinase or EGFP *in vitro* before being treated with BMP-4. Western blot analysis revealed that the phosphorylation of Smad1/5/8 in response to BMP-4 was independent of Smad4 (Fig. 4*A*). In these MEF cultures, the ALP activity induced by BMP-4 was reduced but not eliminated in the Cre-expressing MEFs (Fig. 4*B*). We further examined the role of Smad4 in osteoblastic differentiation using bone marrow stromal/osteoblastic cells prepared from *Smad4*$^{floxed/floxed}$ mice. Again, *Smad4* was deleted *in vitro* by infection with an adenovirus expressing Cre, but expression of ALP was not eliminated in these cells; in fact, ALP expression was still induced by BMP-4 in a dose-dependent fashion in Smad4-deleted cultures (Fig. 4, *C* and *D*). These results suggested that Smad4 is not essential for BMP-induced osteoblastic differentiation but that it may enhance BMP signaling.

Runx2 is essential for osteoblast differentiation and also interacts with R-Smads (30–32). Overexpression of Smad1(DVD) or Runx2 alone induced ALP activity in C2C12 cells, and co-expression of Smad1(DVD) and Runx2 further increased ALP activity (Fig. 5*A*). In contrast, a dominant-negative form of Runx2 blocked ALP induction by Smad1(DVD) (Fig. 5*A*). RNAi knockdown of Runx2 reduced numbers of ALP-positive cells in C2C12 cultures induced by BMPR-IA(Q233D) (Fig. 5, *B* and *C*). Taken together, these findings suggest that phosphorylated R-Smads and Runx2 may cooperatively induce osteoblast differentiation in response to BMPs.

Smad4 Is Involved in Inhibition of Myogenic Differentiation by BMPs—We next examined the roles that the Smad signaling pathway plays in the inhibition of myogenic differentiation by BMPs. Overexpression of Smad1 mutants, including Smad1(DVD), did not inhibit the myogenic differentiation

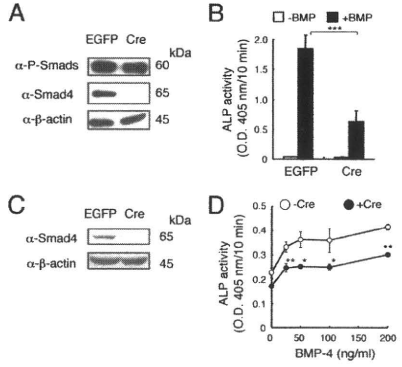

FIGURE 4. **Smad4 is not essential for the osteoblastic differentiation induced by BMP-4.** *A*, MEFs prepared from *Smad4*$^{floxed/floxed}$ were infected with adenovirus expressing Cre recombinase or EGFP. The cells were stimulated for 30 min with 100 ng/ml of BMP-4 to induce phosphorylation of Smad1/5/8. *B*, adenovirus-infected MEFs were treated for 3 days with 100 ng/ml of BMP-4, and ALP activity was determined on day 3. Values are mean ± S.D. ($n = 5$). ***, $p < 0.001$. *C*, Western blot analysis of Smad4-ablated bone marrow stromal/osteoblastic cells prepared from *Smad4*$^{floxed/floxed}$ mice. *D*, dose-dependent induction of ALP activity by BMP-4 in *Smad4*$^{floxed/floxed}$-derived bone marrow stromal/osteoblastic cells infected with Cre-expressing adenovirus or uninfected cells. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$.

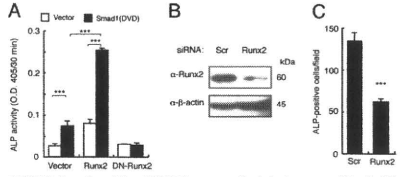

FIGURE 5. **Runx2 and Smad1(DVD) cooperatively induce osteoblastic differentiation of C2C12 cells.** *A*, C2C12 cells were co-transfected with *Smad1(DVD)* and *Runx2* or dominant-negative *Runx2*, and ALP activity was determined on day 3. Values are mean ± S.D. ($n = 3$). ***, $p < 0.001$. *B*, 20 nmol of *Runx2* siRNA or scrambled oligonucleotide was transfected in C2C12 cells, and protein levels were determined by Western blots at 24 h post-transfection. *C*, the siRNA-transfected C2C12 cells were co-transfected with *Bmpr-Ia(Q233D)* and *Smad1*, and ALP activity was determined on day 3. Values are mean ± S.D. ($n = 3$). ***, $p < 0.001$ compared with a scrambled siRNA transfection group.

induced by MyoD. Co-transfection of *Smad4* with *Smad1(DVD)* was required to reduce the number of MHC-positive cells (Fig. 6, *A* and *B*). This role of Smad4 in myogenic differentiation was confirmed in *Smad4*$^{floxed/floxed}$ MEFs. The transcriptional activity of MyoD was suppressed by BMP-4 in MEF cultures infected with a control virus (Fig. 6*C*). In contrast, the basal transcriptional activity of MyoD was increased ~1.4-fold in MEFs infected with the Cre-expressing adenovirus. This increase was not suppressed by BMP-4, suggesting that Smad4 is essential for the suppression of myogenic differentiation by BMPs

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BMP Smads Convert Myoblasts to Osteoblasts

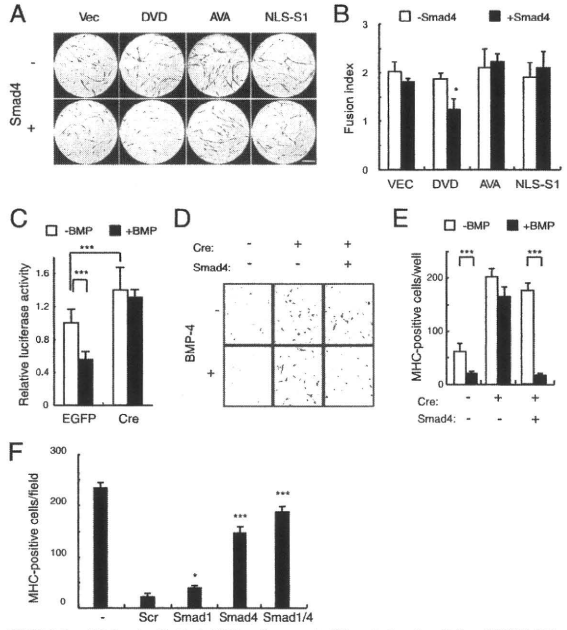

FIGURE 6. **Smad4 is involved in the inhibition of myogenic differentiation.** *A* and *B*, Smad1(DVD) inhibits myogenic differentiation only in the presence of Smad4. C3H10T1/2 fibroblasts were co-transfected with *MyoD* and empty vector (*Vec*), *Smad1(DVD)*, *Smad1(AVA)*, or *NLS-Smad1* (*NLS-S1*) in the absence (*upper panels*) or presence (*lower panels*) of *Smad4* (*A*). Myogenic cells were immunostained for MHC (*red*) on day 5. *Scale bar*, 400 μm. *B*, fusion index was determined in the presence (*closed bars*) or absence (*open bars*) of Smad4. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$. *C*, Smad4 is required for suppression of transcriptional activity of MyoD by BMP-4. MEFs prepared from *Smad4*$^{floxed/floxed}$ were infected with adenovirus expressing EGFP or Cre recombinase, and then the transcriptional activity of MyoD was determined in the presence or absence of 100 ng/ml of BMP-4. Values are mean ± S.D. ($n = 10$). ***, $p < 0.001$. *D* and *E*, Smad4 is essential for inhibition of myogenic differentiation by BMPs. Clonal cell line 16 infected with Cre-expressing or EGFP-expressing control adenovirus was transfected with *MyoD* and stained for MHC on day 5 (*D*). *E*, the numbers of MHC-positive cells were counted in cultures prepared as in *D*. Values are mean ± S.D. ($n = 6$). ***, $p < 0.001$. *F*, 20 nmol of *Smad1* or *Smad4* siRNA or a scrambled oligonucleotide was transfected into C2C12 cells. These cells were treated with 100 ng/ml of BMP-4, and MHC-positive cells were counted on day 5. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$ compared with a scrambled siRNA transfection group.

(Fig. 6*C*). This hypothesis was further confirmed using the floxed *Smad4* MEF cell line, clone 16. Transient transfection of MyoD in clone 16 induced a small number of MHC-positive cells, which were reduced in number by BMP-4 (Fig. 6, *D* and *E*). Infection by Cre-expressing adenovirus not only increased the number of MHC-positive cells, but also maintained this increase in the presence of BMP-4 (Fig. 6, *D* and *E*). Transient transfection of Smad4 in Cre-adenovirus-infected cultures restored the suppression of myogenic differentiation in response to BMP-4 (Fig. 6, *D* and *E*). Moreover, RNAi knockdown of Smad4 increased the number of MHC-positive cells in C2C12 cell cultures treated with BMP-4 (Fig. 6*F*). In contrast, knockdown of Smad1 had minimal effects on these cultures (Fig. 6*F*).

Inhibition of Myogenic Differentiation by Nuclear Smad4—Because Smad4 may translocate into the nucleus as a complex with Smad1/5/8 in response to BMP stimulation, we generated NLS-Smad4 and deletion mutants lacking amino-terminal MH1 (NLS-Smad4(ΔMH1)) and carboxyl-terminal MH2 (NLS-Smad4(ΔMH2)) domains, respectively (Fig. 7*A*). Nuclear localization of these NLS-Smad4 mutants was confirmed, although wild-type Smad4 was mainly detected in the cytoplasm (Fig. 7*B*). Overexpression of full-length NLS-Smad4 suppressed the myogenic differentiation of C3H10T1/2 cells induced by MyoD in a dose-dependent manner (Fig. 7, *C* and *D*, and data not shown). Unexpectedly, NLS-Smad4(ΔMH1), but not NLS-Smad4(ΔMH2), stimulated myogenic differentiation, suggesting that NLS-Smad4(ΔMH1) behaved in a dominant-negative fashion (Fig. 7, *C* and *D*). The Smad4 MH2 domain may thus interact with other molecules essential for inhibition of myogenic differentiation.

Involvement of E4F1 in the Inhibition of Myogenic Differentiation by BMP Signaling—We searched a protein-protein interaction data base that was constructed based on the mammalian two-hybrid method established by the RIKEN group (33) and found several proteins that formed complexes with Smad4. Among these proteins, we focused on E4F1, which contains six zinc fingers and a ubiquitin E3 ligase domain (Fig. 9*A*), because it appeared to be one of the transcription factors principally responsible for inhibition of myogenic differentiation by Smad4.

FLAG-tagged E4F1 was expressed in nuclei, co-localized with NLS-Smad4, and bound to larger amounts of NLS-Smad4 than wild-type Smad4 (Fig. 8, *A* and *B*). Interaction between endogenous Smad4 and FLAG-E4F1 was also detected in C2C12 cells (Fig. 8*C*). NLS-Smad4(ΔMH1), but not NLS-Smad4(ΔMH2), bound to E4F1, confirming that the complex is formed via the MH2 domain of Smad4 (Fig. 8*D*).

Both wild-type E4F1 and E4F1(ΔE3), but not zinc finger mutants, suppressed myogenic differentiation, suggesting that

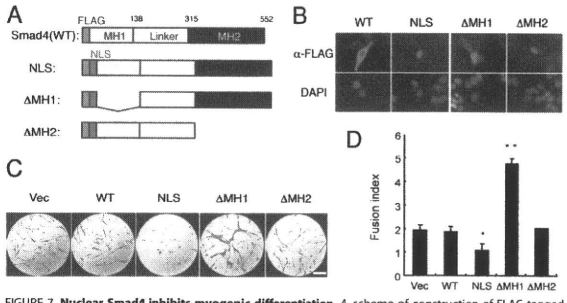

FIGURE 7. **Nuclear Smad4 inhibits myogenic differentiation.** *A*, scheme of construction of FLAG-tagged Smad4 mutants. *B*, cellular localization of FLAG-tagged Smad4 mutants. The cells were immunostained with α-FLAG antibody. *Scale bar*, 25 μm. *C*, effects of Smad4 mutants on myogenic differentiation. C3H10T1/2 cells were co-transfected with *MyoD* and one of the *Smad4* constructs and immunostained for MHC on day 5. *Scale bar*, 400 μm. *D*, fusion index was determined from cultures prepared as in *C*. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$. *DAPI*, 4′,6-diamidino-2-phenylindole.

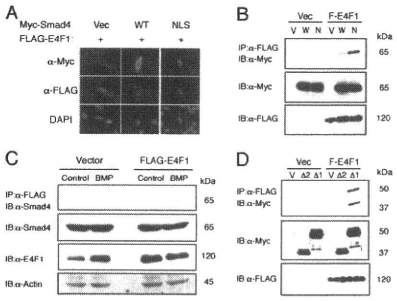

FIGURE 8. **E4F1 inhibits myogenic differentiation in cooperation with nuclear Smad4.** *A*, E4F1 and Smad4 overlapped in nuclei in C2C12 cells. FLAG-tagged *E4F1* and empty vector (*Vec*), *Myc-Smad4* (*WT*), or *Myc-NLS-Smad4* (*NLS*) were cotransfected and stained with α-Myc and α-FLAG antibodies. *B* and *C*, E4F1 interacts with Smad4 *in vivo*. *B*, COS-7 cells were co-transfected with *FLAG-E4F1* and empty vector (*Vec*), *Myc-Smad4* (*WT*), or *Myc-NLS-Smad4* (*NLS*). Whole cell lysates were immunoprecipitated (*IP*) with α-FLAG antibody followed by immunoblotting (*IB*) using α-Myc antibody. *C*, C2C12 cells transfected with *Flag-E4F1* were treated for 1 h without or with 100 ng/ml of BMP-4. Whole cell lysates were immunoprecipitated with M2-agarose beads followed by immunoblotting with antibodies for E4F1, Smad4, and actin. *D*, the MH2 domain of Smad4 is required for interaction with E4F1. The interaction between E4F1 and Smad4 was determined by cotransfection of *Flag-E4F1* and *Myc-NLS-Smad4*(*ΔMH2*) or *Myc-NLS-Smad4*(*ΔMH1*) in COS-7 cells. *DAPI*, 4′,6-diamidino-2-phenylindole.

E4F1 may inhibit myogenesis as a transcription factor rather than as a ubiquitin ligase (Fig. 9*B*). Although *Myogenin* is one of the targets of MyoD and is markedly suppressed by BMP signaling, we could not detect Smad4 or E4F1 binding to the *Myogenin* promoter in response to BMP-4 (supplemental Fig. S2). RNAi knockdown of E4F1 increased the number of MHC-positive C2C12 cells in the presence of BMP signaling (Fig. 9, *D* and *E*). Similar results were obtained using a plasmid-based microRNA expression vector for E4F1 (supplemental Fig. S3). We further examined the role of E4F1 in myogenesis in cell line clone 16 established from *Smad4*$^{floxed/floxed}$ MEFs. Again, deletion of *Smad4* by Cre-adenovirus infection increased the number of MyoD-induced MHC- and myogenin-positive myogenic cells (Fig. 9, *F* and *G*). Co-transfection of *Smad4* and *E4F1* markedly reduced the number of myogenic cells, suggesting that E4F1 acts cooperatively with Smad4 (Fig. 9*F*).

Id1–3 suppress myogenesis and are targets of BMP signaling. Transfection of *E4f1* increased Id1-, Id2-, and Id3-luc activities in C2C12 cells treated with and without BMP-4 (Fig. 9*G*, and data not shown). This stimulation by E4F1 seemed to be Smad4 dependent because the activity was lost by Smad4 ablation and restored by Smad4 overexpression in MEF clone 16 (Fig. 9*G*).

DISCUSSION

In the present study, we examined the molecular mechanisms underlying the conversion of myoblasts by BMPs, allowing their differentiation into osteoblastic cells. It has been suggested that a unique type of intracellular BMP signaling is involved in this conversion, because other inhibitors of myogenic differentiation, such as TGF-β and fibroblast growth factors, do not induce ectopic bone formation *in vivo* or osteoblastic differentiation *in vitro* (12). We found that the inhibition of myogenic differentiation by BMP-4 required treatment for less than 1 h, although induction of osteoblastic differentiation required treatment for more than 9 h. Both activities of BMPs were dependent on the Smad pathway, suggesting that related but distinct mechanisms regulate the conversion of myoblasts into osteoblastic cells. Because we failed to detect cells positive for both MHC and ALP in C2C12 cell cultures treated with BMPs (9, 12), it appeared that osteoblastic differentiation is activated only in immature myoblasts that have not yet initiated myogenic differentiation (34). This hypothesis was confirmed by our preliminary observation that BMPs did not induce ALP activity in mature multinucleated myotubes.[3]

BMP treatment can convert the differentiation pathway of myoblasts into osteoblastic cells and overexpression of constitutively activated BMP type I receptors such as BMPR-IA, BMPR-IB, and ALK2 can have the same effect without requiring the addition of BMPs (35, 36). However, we found that levels of endogenous Smad1 and Smad5 were low in C2C12 cells and that overexpression of wild-type Smad1 was required for induction of osteoblastic differentiation by BMPR-IA(Q233D). These findings suggested that downstream signaling of BMP type I receptors, rather than BMP type II and co-receptors, plays an important role in the conversion of myoblast differentiation. We established a constitutively activated Smad1, Smad1(DVD), to directly examine the role of the Smad pathway without activation of other signaling pathways induced by BMP receptors, such as the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. Serine residues were substituted with aspartic residues in Smad1(DVD) to introduce negative charges in the SVS motif at the carboxyl terminus, the site of phosphorylation by type I BMP receptors. These substitutions may induce conformational changes and stimulate interaction with coactivators such as p300, OAZ, and Runx2 (11). Indeed, Smad1(DVD) was directly recognized by the α-phospho-Smad1/5/8 antibody, suggesting that its three-dimensional structure is similar to that of native Smad1 phosphorylated by the receptors. We found that co-transfection of *Smad1(DVD)* with *Smad4* was capable of inducing osteoblastic differentiation of C2C12 myoblasts, and this induction was not inhibited by co-expression with Smad7, suggesting that Smad1(DVD) induces activation downstream of BMP type I receptors without activating endogenous BMPs or receptors. However, the ALP activity induced by Smad1(DVD) and Smad4 was lower than that induced by cotransfection of a constitutively activated BMPR-IA receptor and wild-type Smad1, although it was higher than that induced by BMPR-IA(Q233D) alone or Smad1(EVE), in which serine residues had been substituted with glutamic acids instead of aspartic acids.[3] These findings suggested that native phosphorylated Smad1 may have higher affinity for the coactivators required for osteoblastic differentiation than Smad1(DVD) or Smad1(EVE). This hypothesis will require further testing.

The Smad signaling pathway was also involved in the inhibition of myogenic differentiation. In contrast to osteoblast differentiation, however, this inhibitory activity of the Smad pathway appeared to be mainly dependent on Smad4 rather than R-Smads. In particular, the nuclear-targeted Smad4 markedly suppressed myogenic differentiation, although overexpression of NLS-Smad4 did not induce osteoblastic differentiation,[3] suggesting that Smad4 in complex with R-Smads inhibits myogenic differentiation after translocation from the cytoplasm to the nucleus in response to BMP stimulation. Because Smad4 is a common Smad among the TGF-β superfamily members, it may also be involved in mediating the effects of other myogenic inhibitors, such as TGF-βs, myostatin, and activin (37, 38).

The MH1 and MH2 domains of Smads have been shown to be involved in DNA binding and interaction with other proteins, respectively (39). Our deletion analysis suggested that nuclear Smad4 may interact with other transcriptional factor(s) and recruit them to the target DNA sequences via the MH2 and MH1 domains, respectively, to suppress myogenesis. This hypothesis was further supported by the finding of

[3] J. Nojima, T. Takada, and T. Katagiri, unpublished data.

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2010/03/15/M109.028019.DC1.html

BMP Smads Convert Myoblasts to Osteoblasts

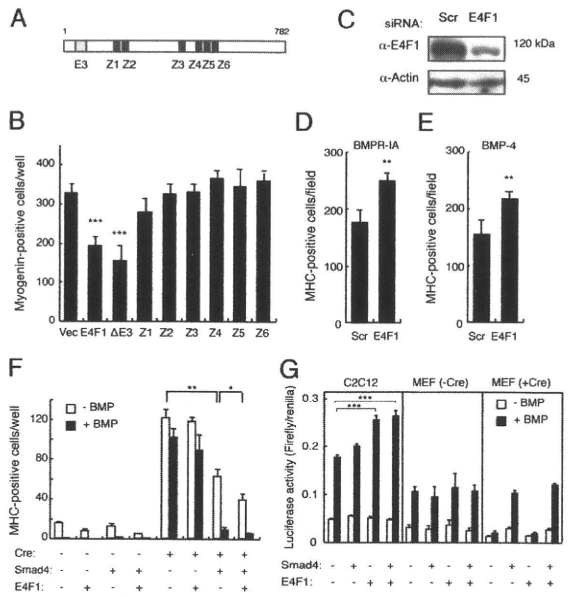

FIGURE 9. **E4F1 is involved in the BMP-induced inhibition of myogenesis.** *A*, schematic structure of E4F1. *E3*, an E3 ubiquitin ligase domain; *Z*, zinc fingers. *B*, overexpression of E4F1 inhibits myogenic differentiation. C3H10T1/2 cells were co-transfected with *MyoD* and wild-type *E4F1*, *E4F1*(*ΔE3*), or a zinc finger mutant *E4F1* and immunostained for myogenin on day 3. Values are mean ± S.D. ($n = 3$). ***, $p < 0.001$. *C*, 20 nmol of *E4F1* siRNA or scrambled oligonucleotide was transfected in C2C12 cells, and protein levels were determined by Western blots at 24 h post-transfection. *D* and *E*, the siRNA-transfected C2C12 cells were co-transfected with *Bmpr-Ia*(*Q233D*) (*D*) or treated with 100 ng/ml of BMP-4 (*E*), and MHC-positive cells were counted on day 5. Values are mean ± S.D. ($n = 3$). **, $p < 0.01$ compared with each scrambled siRNA transfection group. *F*, E4F1 suppresses myogenesis cooperatively with Smad4. *Smad4*$^{floxed/floxed}$-derived clonal cell line 16 was infected with or without a Cre-expressing adenovirus. The cells were transfected with or without *Smad4* in the presence or absence of Smad4, cultured with or without 100 ng/ml of BMP-4, and stained for MHC on day 3. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$. *G*, E4F1 stimulates *Id1* expression. C2C12 cells and MEF clone 16 infected with Cre adenovirus were transfected with *Id985-luc*, *E4F1*, and *Smad4* and were then cultured in the presence or absence of 50 ng/ml of BMP-4. Luciferase activity was determined on day 1. Values are mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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stimulation of myogenic differentiation by NLS-Smad4(ΔMH1); this mutant Smad4 lacking DNA-binding activity may quench the transcriptional activity of the complex via the MH2 domain. It also appeared that a component of the Smad4 complex, interacting through the MH2 domain, is critical for inhibition of myogenic differentiation in response to BMPs. In the present study, we identified E4F1 as one of the components of the Smad4 complex in the nucleus, interacting through the MH2 domain. E4F1 is a zinc finger DNA-binding protein, identified as a cellular target of viral oncoproteins and shown to regulate the cell cycle (40–42). Our findings indicated that overexpression of E4F1 inhibited myogenic differentiation cooperatively with Smad4. Moreover, RNAi knockdown of E4F1 prevented the inhibition of myogenic differentiation by BMP signaling. Although E4F1 was recently shown to act as a ubiquitin E3 ligase of p53 (43), our findings indicated that deletion of the ubiquitin E3 ligase domain from E4F1 still allowed inhibition of myogenic differentiation. However, all of the zinc finger structures of E4F1 seemed to be important for this inhibitory activity. Taken together, these findings suggest that Smad4, which undergoes nuclear translocation in response to BMP stimulation, may interact with E4F1 in the nucleus to suppress myogenic differentiation as a transcription factor, independent of its ubiquitin E3 ligase activity. Recently, it was reported that Smad4 regulates the processing of pri-microRNA into mature microRNA in response to BMP-2 treatment (44). The direct target gene(s) of the complex still needs to be identified. It is interesting to note that loss-of-function mutations of p53 and Smad4 were identified in some tumors, suggesting that mutations in the Smad4-E4F1-p53 axis might play a role in tumorigenesis (45, 46).

We found that E4F1 stimulated the expression of *Id1–3* in the presence of Smad4. Id proteins inhibit myogenesis and are targets of BMP signaling. Recently, insufficient skeletal muscle repair was reported in $Id1^{+/-}Id3^{-/-}$ mice after muscle injury (47). BMP signaling may also up-regulate *Id* expression in healing muscle tissue (47). Because expression of *Ids* leads to cell cycle progression, the E4F1-induced Ids may suppress myogenic differentiation and maintain myoblast proliferation. Further studies are needed to elucidate the physiological roles of Smads and E4F1 in muscle development and regeneration *in vivo*.

In the present study, we obtained an unexpected finding related to Smads. BMP-induced osteoblastic differentiation was not completely blocked in the *Smad4*-deleted MEFs. There are some possible explanations for this finding: 1) undetectable levels of Smad4 still remained in the MEFs expressing Cre recombinase, 2) an alternative pathway, including a novel Co-Smad, transduced BMP signaling, or 3) Smad4 is not essential for the osteoblastic differentiation induced by BMPs. Recently, evidence has been presented that bone and cartilage tissues were formed during development in the absence of functional Smad4 in mice, although such mice exhibited abnormalities (48). Deletion of *Smad4* in mouse mature osteoblasts using a Cre-loxP system significantly reduced bone volume and osteoblast function *in vivo*, but they still had bone tissues and osteoblasts (48). Further study will be required to elucidate the roles of Smad4 in bone metabolism.

In conclusion, we found that the Smad-dependent pathway regulates both the inhibition of myogenic differentiation and the induction of osteoblastic differentiation induced by BMPs. The introduction of negative charges at the carboxyl terminus of Smad1 may play an important role in the induction of osteoblast differentiation in response to BMPs. In contrast, nuclear Smad4, rather than R-Smad, and E4F1, a novel partner of nuclear Smad4, are responsible for the inhibition of myogenic differentiation by BMPs.

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