TABLE 3. Prevalence of OPLL in General Population in Japan

Reporter	Location of Survey	Subjects (M, F)	Age of Subjects (yr)	OPLL (n)	Incidence of OPLL (%)
Ikata and Tezuka 1979[24]	Tokushima	696 (330, 366)	>20	21	3.0
Ohtani *et al* 1980[25]	Yaeyama	1046 (578,468)	>20	21	2.0
Yamauchi *et al* 1982[26]	Kamogawa	787 (408, 379)	>40	20	2.5
	Kofu	383 (169, 214)	>40	13	3.4
Sakou and Morimoto 1982[27]	Kagoshima	585 (195, 390)	>30	11	1.9
Ohtsuka *et al* 1984[28]	Yachiho	1058 (440, 618)	>50	34	3.2
Ikata *et al* 1985[29]	Tokushima	415 (122, 293)	>30	18	4.3

OPLL indicates ossification of the posterior longitudinal ligament.

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glycoprotein, is thought to produce inorganic pyrophosphate, a major inhibitor of calcification and mineralization. Some researchers reported the association of the human *NPPS* with OPLL. Now, nationwide DNA analysis of candidate gene for the sibpair of OPLL is proceeding by the Investigation Committee on Ossification of the Spinal Ligaments of Japanese Ministry of Public Health, Labour, and Welfare.

NATURAL HISTORY

The Investigation Committee on Ossification of the Spinal Ligaments of the Japanese Ministry of Public Health, Labour, and Welfare performed the first national survey of OPLL in 1975, and 2142 OPLL patients were registered. On the basis of the results of this survey, the average age of onset was 51.2 years in men and 48.9 years in women. Ninety-five percent of patients had some clinical symptoms, but 5% of patients were free of symptoms. As many as 16.8% of patients needed assistance in activities of daily living, 5.4% of patients showed a rapid aggravation of symptoms, and 11.4% of patients showed a chronic aggravation. A total of 23% of patients had a history of trauma to the cervical region. Trauma in the cervical spine may have precipitated the onset of symptoms, which, in some cases, included quadriparesis.

TABLE 4. Relationship Between the Share of Identical HLA Haplotypes and Existence of OPLL in 61 Siblings

	Two Strands Identical (n = 19)	One Strand Identical (n = 21)	Not Identical (n = 21)
OPLL	10 (53%)	5 (24%)	1 (5%)

The percentage represents the rate of existence of OPLL on radiography and computed tomography in each group. The rate of existence of OPLL in the group with 2 identical strands is significantly higher than other 2 groups. $P < 0.05$.

OPLL indicates ossification of the posterior longitudinal ligament; HLA, human leukocyte antigen.

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However, the prevalence of trauma that caused symptoms was only 15% in the retrospective study. In the prospective investigation for 368 OPLL patients without myelopathy at the time of initial consultation, only 6 patients (2%) subsequently developed trauma-induced myelopathy.[40] A total of 112 OPLL patients treated conservatively were observed for 1 to 16.9 years. Progression of ossification in length was demonstrated in 24% of patients and, in thickness, in 13% of patients in the group. Progression of ossification did not always lead to exacerbation of symptoms.[41]

In our personal study, a total of 450 patients, averaging 74.6 years of age at last evaluation, were prospectively observed for an average of 17.6 years (10–30 years) to discern the natural history of disease progression.[42,43] Myelopathy was originally recognized in 127 patients, 91 of whom were managed surgically. The remaining 36 patients with myelopathy were treated nonoperatively, and an increase in myelopathy was observed in 23 (65%) of these individuals. For the 323 patients without original myelopathy, 64 (20%) became myelopathic during the follow-up interval. The Kaplan-Meier estimate of myelopathy-free survival among patients without myelopathy at the first visit was 71% at 30-year follow-up (Figure 1). Forty-five patients with more than 60% of the spinal canal compromised by OPLL were all myelopathic. The range of motion of the cervical spine was calculated by dynamic radiography. The range of motion was significantly larger in patients with myelopathy and with less than 60% of the canal compromised with OPLL. Although myelopathy was recognized in all patients with more than 60% of the spinal canal compromised by OPLL, small OPLL at first examination rarely developed to large OPLL with more than 60% stenosis during the follow-up. Therefore, one cannot simply say that myelopathy develops with OPLL. Dynamic factors (range of motion) appear to be very important for the evolution of myelopathy in patients with less than 60% of the canal compromised with OPLL. The study for risk factors for development of myelopathy in patients with OPLL is important. A multicenter cohort study for radiographic predictors for development of myelopathy in patients with OPLL by the

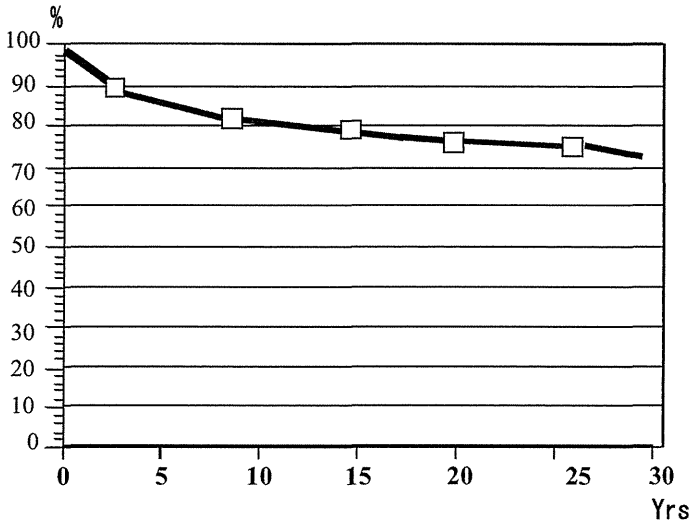


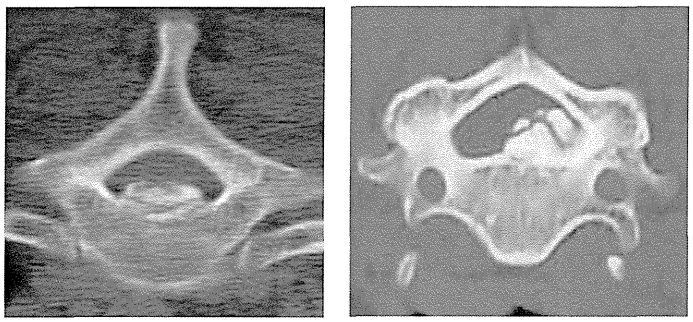
Figure 1. Myelopathy-free rate calculated by Kaplan-Meier method in patients with ossification of the posterior longitudinal ligament. Reprinted with permission from Yonenobu *et al. OPLL*. 2nd ed. Tokyo, Japan: Springer–Verlag; 2006:14.

Investigation Committee on Ossification of the Spinal Ligaments of the Japanese Ministry of Public Health, Labour, and Welfare was performed.[44] This study showed that more than 60% spinal canal stenosis by OPLL and lateral deviated–type OPLL on CT (Figure 2) were radiographic risk factors for development of myelopathy in patients with OPLL.

MEASUREMENT TOOL AND IMAGING DIAGNOSIS

Plain Radiographs

OPLL was mostly found at the C4, C5, and C6 levels. The level with the maximal thickness of OPLL was often C5. OPLL in the cervical spine may be radiographically classified into 4 types, based on the findings on a lateral radiograph: continuous, mixed, segmental, and other (Figure 3). In the nationwide survey in Japan, the segmental type was recognized in 39%, continuous in 27%, mixed in 26%, and other in 7%. The sagittal diameter of the spinal canal is measured as the distance from the posterior aspect of the vertebral body



(A) **Central type** (B) **Lateral deviated type**

Figure 2. Ossification of the posterior longitudinal ligament pattern on computed tomographic examination. (**A**), Central type; (**B**), lateral deviated type. Reprinted with permission from Matsunaga *et al. Spine* 2008;24:2648–50.

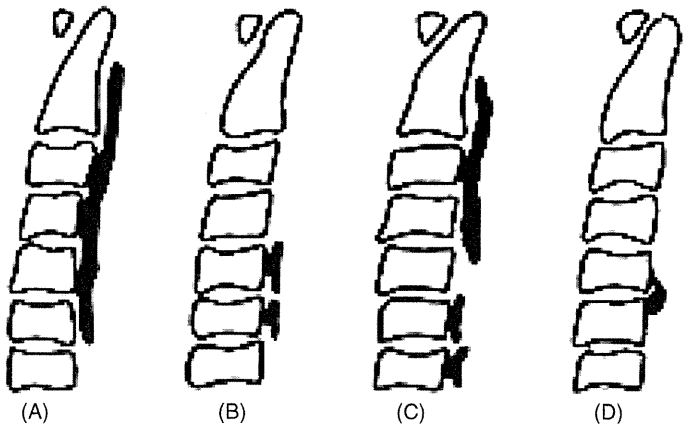


Figure 3. Classification of ossification of the posterior longitudinal ligament by the members of the Investigation Committee on the Ossification of the Spinal Ligaments of the Japanese Ministry of Health, Labour, and Welfare. (**A**), Continuous type; (**B**), segmental type; (**C**), mixed type; (**D**), unclassified type. Reprinted with permission from Yonenobu et al. *OPLL*. Tokyo, Japan: Springer–Verlag; 1997:12.

to the anterior edge of the base of the spinous process on the lateral view. This distance is regarded as the anteroposterior diameter of the cervical canal. The percentage of the thickness of the ossification to the anteroposterior diameter of the spinal canal is then regarded as the spinal canal stenosis rate. The maximum spinal canal stenosis rate, as determined at the thickest point of the ossified area, was 38% on average in cases with myelopathy and 27% in cases without myelopathy. The thickness of ossification was not always associated with the degree of neurologic dysfunction, that is, paralysis. Even if ossification is severe within the canal, neurologic symptoms are sometimes mild.

Computed Tomography

CT provides an excellent axial view of the spinal canal, yielding valuable information concerning the ossified area and its median or paramedian location. Three-dimensional CT studies provide excellent documentation of the 3-dimensional morphology of OPLL involving the cervical spinal canal.[45]

Magnetic Resonance Imaging

OPLL is usually visible as a signal-free or low-signal area on magnetic resonance imaging studies. However, hypointense, isointense, and hyperintense areas may also be noted in portions of ossified tissue, representing small medullary cavities actively involved in bone marrow production. Hypertrophy of the posterior longitudinal ligament has been evaluated using magnetic resonance imaging. A dilated signal-free area in the posterior aspect of the vertebral bodies is visible in some without OPLL. A high-intensity area in the spinal cord on the T2-weighted images, observed in 25% to 45% patients with OPLL, likely corresponds to irreversible intrinsic changes within the cord, that is, myelomalacia.

CONCLUSION

OPLL is still a mysterious disease. However, recent clinical and genetic studies have been clarifying natural history and

pathogenesis of this disease. The knowledge of natural history of OPLL would be useful for treating OPLL appropriately. Elucidation of pathogenetic genes of OPLL will introduce a new approach for the management of OPLL.

➢ Key Points

- ❑ The prevalence of OPLL of the cervical spine in the general Japanese population is 1.9% to 4.3%.
- ❑ Pathogenetic candidate genes of development of OPLL have been reported, and the pathogenetic genes will be clarified in the future.
- ❑ Natural history of OPLL has been shown by prospective study of the Investigation Committee on the Ossification of the Spinal Ligaments of the Japanese Ministry of Health, Labour, and Welfare.

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Pathogenic Mutation of ALK2 Inhibits Induced Pluripotent Stem Cell Reprogramming and Maintenance: Mechanisms of Reprogramming and Strategy for Drug Identification

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Key Words. Induced pluripotent stem cells • Reprogramming • Pluripotency • Experimental models

ABSTRACT

Fibrodysplasia ossificans progressiva (FOP) is a rare congenital disorder characterized by progressive ossification of soft tissues. FOP is caused by mutations in activin receptor-like kinase 2 (ALK2) that cause its constitutive activation and result in dysregulation of BMP signaling. Here, we show that generation of induced pluripotent stem cells (iPSCs) from FOP-derived skin fibroblasts is repressed because of incomplete reprogramming and inhibition of iPSC maintenance. This repression was mostly overcome by specific suppression of ALK2 expression and treatment with an ALK2 inhibitor, indicating that the inhibition of iPSC generation and maintenance observed in FOP-derived skin fibroblasts results from constitutive activation of ALK2. Using this system, we identified an ALK2 inhibitor as a potential candidate for future drug development. This study highlights the potential of the inhibited production and maintenance of iPSCs seen in diseases as a useful phenotype not only for studying the molecular mechanisms underlying iPS reprogramming but also for identifying drug candidates for future therapies. STEM CELLS *2012;30:2437–2449*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP) is a congenital disorder of progressive and widespread postnatal ossification of soft tissues and muscles [1–3]. Severe debilitation, reduced life expectancy due to joint fusion, and restrictive ventilatory impairment with thoracic involvement are major symptoms of this disease. Patients with FOP have gradual worsening of pulmonary function and eventually die by 40 due to respiratory failure if they do not receive the appropriate support. There is no effective therapy for preventing the ectopic ossification associated with FOP. Recent studies have revealed that this disorder is caused by mutations in Activin A receptor type I, the gene encoding the bone morphogenetic protein (BMP) type I receptor activin receptor-like kinase 2 (ALK2) [4–9]. The most common mutation is R206H, which is thought to alter ALK2 and confer constitutive activity to the mutant receptor. Mesenchymal cells derived from primary teeth of FOP patients showed elevated basal expression of RUNX2 and alkaline phosphatase (AP), which are involved in bone formation [4]. These data suggest that the dysregulation of BMP signaling seen in FOP patients results in ectopic expression of osteogenesis-related genes and aberrant ossification. Several other mutations in ALK2, such as G356D, underlie phenotypic variations of FOP and these also alter ALK2 and confer constitutive activity to the mutant receptor [10]. The weaker kinase activity of ALK2 (G356D) compared to that of ALK2 (R206H) suggests that clinical variation is due to differences in the bioactivity of ALK2 mutants [11].

Induced pluripotent stem cells (iPSCs) derived from patients with incurable diseases represent a powerful tool not

Author contributions: T.E.: designed all experiments and edited the manuscript; M.H., Y.Y., Y.T., and T.K.: performed the experiments, analyzed data, and edited the manuscript; Y.H.: designed and performed part of the drug candidate experiments and edited the manuscript; N.F.: produced and provided Sendai virus vectors and edited the manuscript; H.H.: designed and performed part of the shRNA experiments and edited the manuscript; Y.N., H.F., and N.H.: provided the patient samples and data and edited the manuscript; T.E and Y.T.: wrote and prepared the manuscript and edited the manuscript.

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only for biomedical research but also for investigating the effects of drugs on patient-derived cells [12–16]. These cells are derived from differentiated somatic cells and functionally resemble embryonic stem cells (ESCs) [17]. This process, known as reprogramming, is triggered by the expression of four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc, which are the same core factors underlying pluripotency in ESCs [17, 18]. This reprogramming process changes a cell's gene-expression profile from that of a somatic cell back to that of a pluripotent state [17]. It is well-known that iPSCs derived from somatic cells harboring pathogenic gene mutations represent the cellular phenotype of the disease [19–21].

Investigations of the process for generating iPSCs are valuable for understanding the molecular mechanisms underlying cellular reprogramming. Recent knockout-mouse studies have identified several genetic mutations that modify the efficiency of iPSC generation. For example, iPSCs can be generated with higher efficiency from *p53*- and *Ink4a/Arf*-null fibroblasts than from normal fibroblasts [22, 23]. A mutation in p21, which is a molecule involved in downstream p53 signaling, partially mimics this phenotype, suggesting that activation of p53 and Ink4a/Arf signals can inhibit cellular reprogramming. These results raise the notion that some genetic mutations underlying human diseases also affect the reprogramming processes and eventually abolished iPSC generation. However, it is still unclear how pathogenic gene mutations affect the cellular reprogramming required for the generation and maintenance of human iPSCs.

Here, we studied disease-derived iPSCs to elucidate how pathogenic gene mutations affect cellular reprogramming. We showed that iPSC generation from FOP-derived skin fibroblasts is repressed. The few FOP-derived iPSCs that we managed to isolate could not be maintained because they differentiated spontaneously into mesodermal and endodermal lineages. We showed that repression of iPSC generation results from inefficient reprogramming of FOP-derived fibroblasts and inhibition of iPSC maintenance. This repression was mostly overcome by specific suppression of ALK2 expression and treatment with an ALK2 inhibitor. Using this system in combination with in silico chemical library screening, we identified an ALK2 inhibitor as a potential drug candidate for future therapeutic applications. The inefficient production of iPSCs is a useful disease phenotype not only for understanding the mechanism of reprogramming but also for identifying drug candidates for future therapies.

Materials and Methods

Generation and Detection of Sendai Virus Vector

The Sendai virus (SeV) carrying Oct3/4, Sox2, Klf4, and c-Myc were generated as described previously [24]. To detect SeV genome, nested RT-PCR was performed. The sequences of primers and amplification conditions are listed in supporting information Table S1.

Cell Culture and iPSC Generation with SeV Vector

Fibroblasts from FOP patients and healthy volunteers were generated from explants of skin biopsy following informed consent under protocols approved by the ethics committee assigning authors. Skin samples were minced and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. After the fibroblast appeared, it was expanded for iPSC induction.

iPSCs were generated from human skin-derived fibroblasts as described previously [24]. Cells (5×10^5) of human fibroblast cells per well of six-well plate were seeded 1 day before infection and then were infected with SeV vectors at a multiplicity of infection of 3. Seven days after infection, fibroblasts infected were harvested by trypsin and replated at 5.4×10^4 cells per 60 mm dish on the mitomysin C (MMC)-treated mouse embryonic fibroblast (MEF) feeder cells. Next day, the medium was replaced in human iPSC medium. Thirty days after infection, colonies were picked up and recultured again in human iPSC medium.

Maintenance of Human iPSCs

Human iPSCs were maintained on MMC-treated MEF feeder cells in human iPS medium containing DMEM/F12 (SIGMA) supplemented with 20% KNOCKOUT serum replacement (Invitrogen), 2 mM L-glutamine, 1×10^{-4} M nonessential amino acids (SIGMA), 1×10^{-4} M 2-mercaptoethanol (SIGMA), 0.5% penicillin and streptomycin (Nacalai Tesque, Kyoto, Japan, http://www.nacalai.co.jp), and 5 ng/ml basic fibroblast growth factor (Wako, Osaka, Japan, http://www.wako-chem.co.jp/). In some experiment, ALK2 inhibitors such as LDN-193189 (STEMGNT, Cambridge, MA, http://www.stemgent.com/) and Dorsomorphin (DM; SIGMA) were added into the human iPS medium. DiPS used as a control iPSC line was kindly gifted by DNAVEC Corporation (Tsukuba, Japan, http://www.dnavec.co.jp/en/index.html).

Chemical Library Screening

We extracted known inhibitors for homologous kinases including BMP receptor family from the CHEMBL database to determine the queries (https://www.ebi.ac.uk/chembldb/). The CHEMBL database, which contains 1,118,865 compounds and 4,668,202 activity data, is provided by European Bioinformatics Institute. We constructed a search engine to retrieve ALK family inhibitors and BMP inhibitors from the CHEMBL database. Using the search engine, 236 known kinase inhibitors were obtained. Finally, 153 compounds were selected from commercially available databases system and purchased (Namiki Shoji Co., Ltd., http://www.namiki-s.co.jp/english/; Kishida chemical Co., Ltd., http://www.kishida.co.jp/english/index.html). Unavailable seven compounds known as ALK2 inhibitor (Thomson Reuters Integrity^{SM}) were prepared by ourselves. In total, 160 bioactive chemical compounds were evaluated by cell-based inhibition assay of BMP signaling. Other materials and methods are described in supporting information Supplemental Materials and Methods.

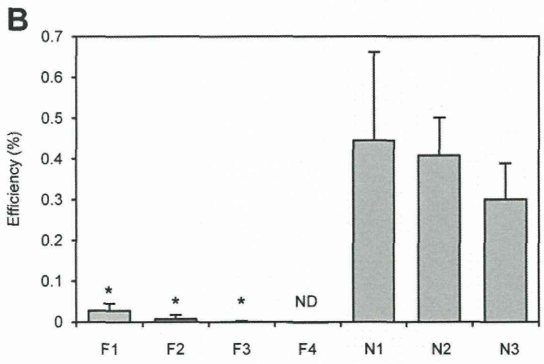
Results

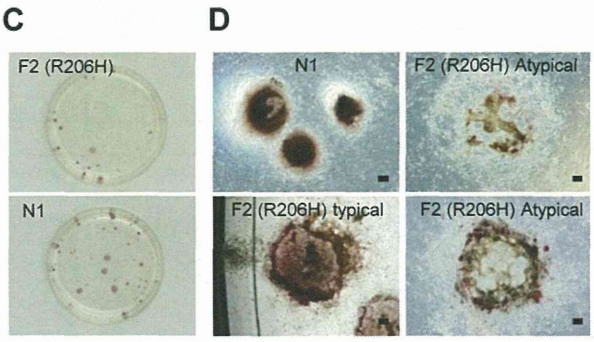
Generation of iPSCs from FOP-Derived Skin Fibroblasts

We attempted to generate iPSCs from skin fibroblasts from four patients with FOP and three healthy volunteers by the SeV method (Fig. 1A) [24–26]. Three patients had the common R206H mutation of ALK2 and the remaining patient had the G356D mutation (supporting information Fig. S1A) [10]. The SeV method is suitable for establishing disease-specific iPSCs because it is highly efficient, does not involve integration, and the SeV is easy to remove. The frequency of iPSC colony formation from FOP-derived fibroblasts was significantly lower than that from the normal controls (Fig. 1B, 1C). Almost all colonies generated from FOP-derived fibroblasts exhibited atypical morphologies compared to controls (Fig.

A

Patients			Diagnosis	Mutation
No.	Age	Sex		
F1	18	M	FOP	R206H
F2	59	F	FOP	R206H
F3	22	M	FOP	R206H
F4	66	M	FOP	G356D
N1	48	M	Healthy	-
N2	54	M	Healthy	-
N3	48	M	Healthy	-





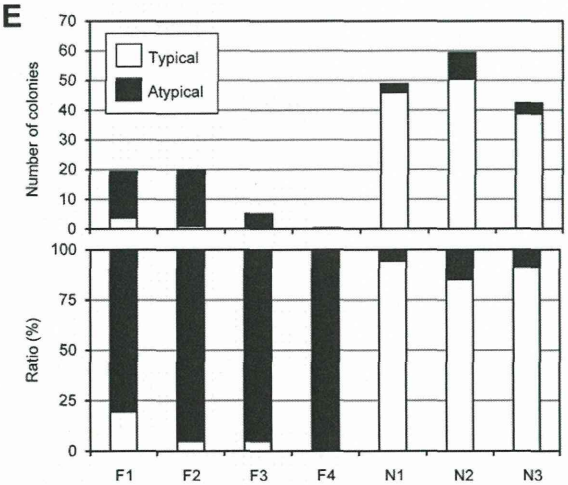


Figure 1. Generation of induced pluripotent stem cells (iPSCs) from patients with FOP. **(A):** Summary of patients with FOP and healthy volunteers. **(B):** Efficiency of generation of the iPSCs from FOP patients and healthy volunteers. iPSC colonies were identified on day 30 of induction by the appearance of alkaline phosphatase-positive (AP^+) colonies with undifferentiated colony morphology (typical iPSC morphology). The efficiency of iPSC generation from FOP-derived fibroblasts is substantially lower than that from healthy volunteers (controls). The data are means ± SD of three independent experiments. A one-way ANOVA followed by Tukey's multiple comparison test was performed to evaluate differences between groups. *, $p < .05$, when compared with each values of fibroblast N1, N2, and N3. **(C):** AP-staining of iPSC colonies in 60 mm dishes. **(D):** Colony morphology of iPSCs. AP^+ colony with undifferentiated colony morphology was counted as a typical colony. Typical colonies were mostly observed in control cultures (upper left). Some typical colonies were detected in cultures derived from FOP patients (lower left). Atypical colonies are defined as those where only the center of the colony was AP-positive (upper right) or those where only the periphery of the colony was AP-positive (lower right). Scale bars = 200 μm. **(E):** The number and percentage of typical and atypical FOP-derived and control iPSC colonies. Almost all FOP-derived colonies exhibited an atypical morphology. Abbreviations: FOP, fibrodysplasia ossificans progressive; ND, not detected.

1D, 1E). Selected colonies of FOP-derived fibroblasts did not expand after several passages, exhibited a flat morphology, and disappeared. It is noted that these morphological changes are very similar to those observed in the induction of iPSC differentiation.

Treatment with ALK2 Inhibitors Can Restore the Ability to Generate and Maintain FOP-Derived iPSCs

In FOP, the mutant ALK2 results in dysregulated BMP signaling via its constitutive activation [3, 5, 9]. We next examined the effects of ALK2 inhibitors on the generation and maintenance of iPSCs. Treatment with the ALK2 kinase inhibitor, LDN-193189 (LDN) [27], restores the colony formation capacity of iPSCs in dose-dependent manner (Fig. 2A). DM [28], another inhibitor for ALK2, also improves the efficiency of colony formation but not to the same extent as that by LDN. LDN enhances the generation of iPSCs from fibroblast F2 cells harboring the R206H mutation as well as from fibroblast F4 cells harboring the G356D, but with lower efficiency (Fig. 2A).

In addition to improving the efficiency of colony formation, treatment with ALK2 inhibitors allows iPSC colonies to be maintained continuously without morphological alteration. The iPS colonies formed in the presence of LDN and DM exhibited a typical morphology (Fig. 2B and supporting information Fig. S1B). Individual colonies were cultured and maintained in the presence of LDN. Absence of the SeV DNA fragment following amplification with nested primers indicated that SeV was completely removed from the iPSC lines (Fig. 2C) [24]. RT-PCR and immunostaining analyses of these FOP-derived iPSC lines revealed that they expressed a set of markers typical of pluripotent cells (Fig. 2C, 2D) [17, 18]. To confirm the pluripotency of FOP-derived iPSC lines, we transplanted them into the subcutaneous tissues of the immunodeficient mice. Eight to twelve weeks after injection, FOP-derived iPSC lines tested formed teratomas that contained derivatives of all three germ layers (Fig. 2E and supporting information Fig. S2). Immunoblot analysis of phosphorylated Smad1/5/8, which are downstream molecules of ALK2 signaling, indicated that ALK2 kinase activity was higher in FOP-derived iPSCs than in controls (supporting information Fig. S3). We also demonstrated Smads dephosphorylation following treatment with ALK2 inhibitors thus confirming the ability of these inhibitors to suppress ALK2 activity (supporting information Fig. S3).

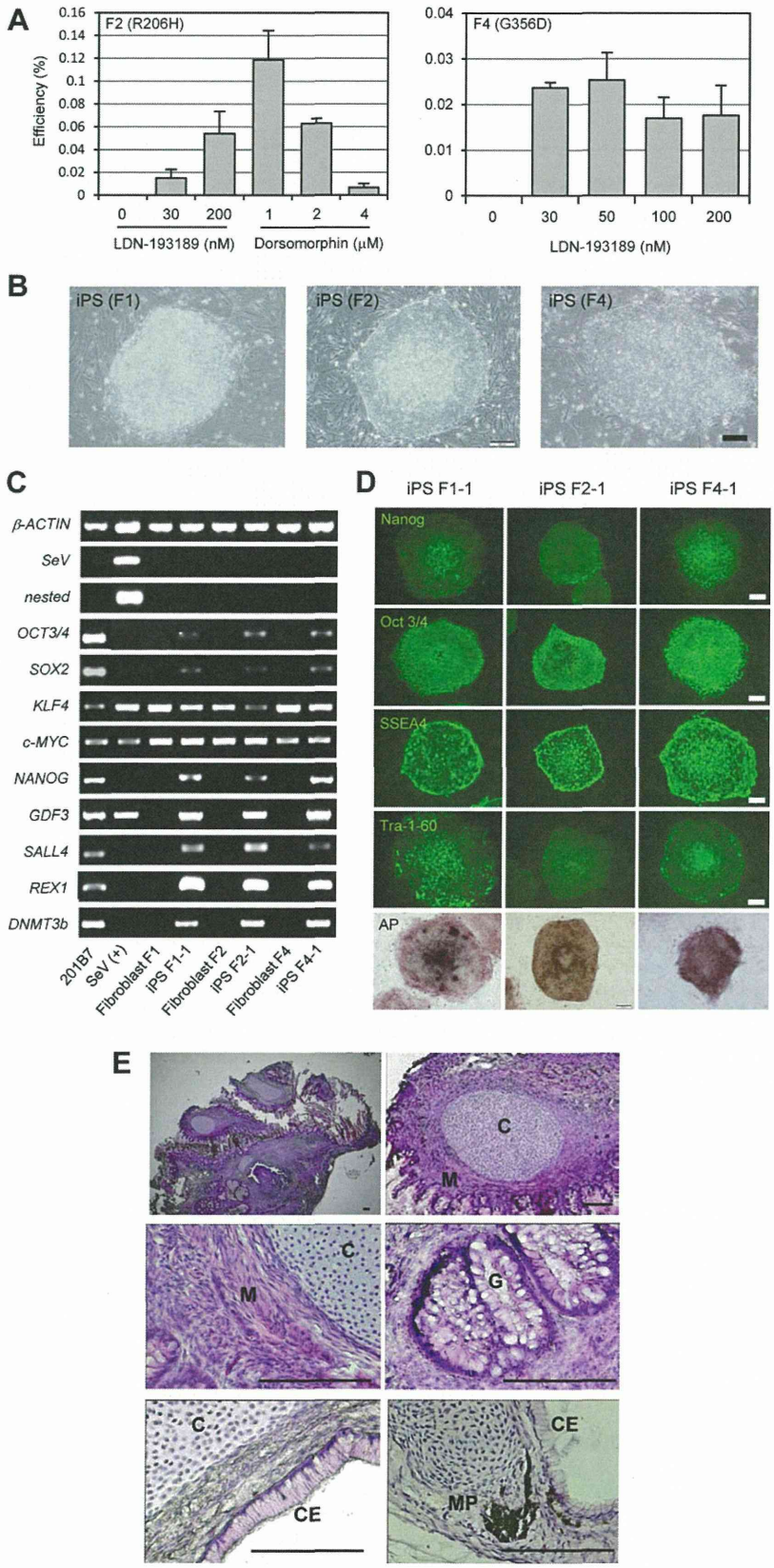


Figure 2. Treatment with activin receptor-like kinase 2 (ALK2) inhibitors can restore the generation and maintenance of iPSCs from fibrodysplasia ossificans progressive (FOP) fibroblasts. **(A):** Improvement of iPSC colony generation by treatment with the ALK2 inhibitors, LDN-193189 (LDN) and Dorsomorphin. FOP-derived fibroblasts were treated with the inhibitors from day 8 to day 30 of iPSC inductions. iPS colonies were identified by the appearance of AP^+ colonies with undifferentiated colony morphology (Fig. 1D). Left and right panels: F2 and F4 patients, respectively. **(B):** Phase-contrast picture of FOP-derived iPSCs generated from individual patients and treated with LDN. Scale bar = 200 μm. **(C):** RT-PCR analysis of SeV and human embryonic stem cell markers. The iPSC lines F1-1, F2-1, and F4-1 are derived from patients, F1, F2, and F4, respectively. 201B7: control human iPSC line. SeV(+): SeV-infected fibroblasts. **(D):** Immunofluorescence and AP staining of FOP-derived iPSC lines for pluripotency markers. Scale bars = 200 μm. **(E):** Tissue morphology of the hematoxylin and eosin-stained representative teratoma derived from FOP-derived iPSC line F2-3. The descendants of three germ layers are observed in the teratoma. G: gut-like structure (endoderm); C: cartilage (mesoderm); M: muscle tissue (mesoderm); CE: cuboidal epithelium (ectoderm); MP: melanin pigment (ectoderm). Scale bars = 200 μm. Abbreviations: AP, alkaline phosphatase; iPS, induced pluripotent stem; SeV, Sendai virus.

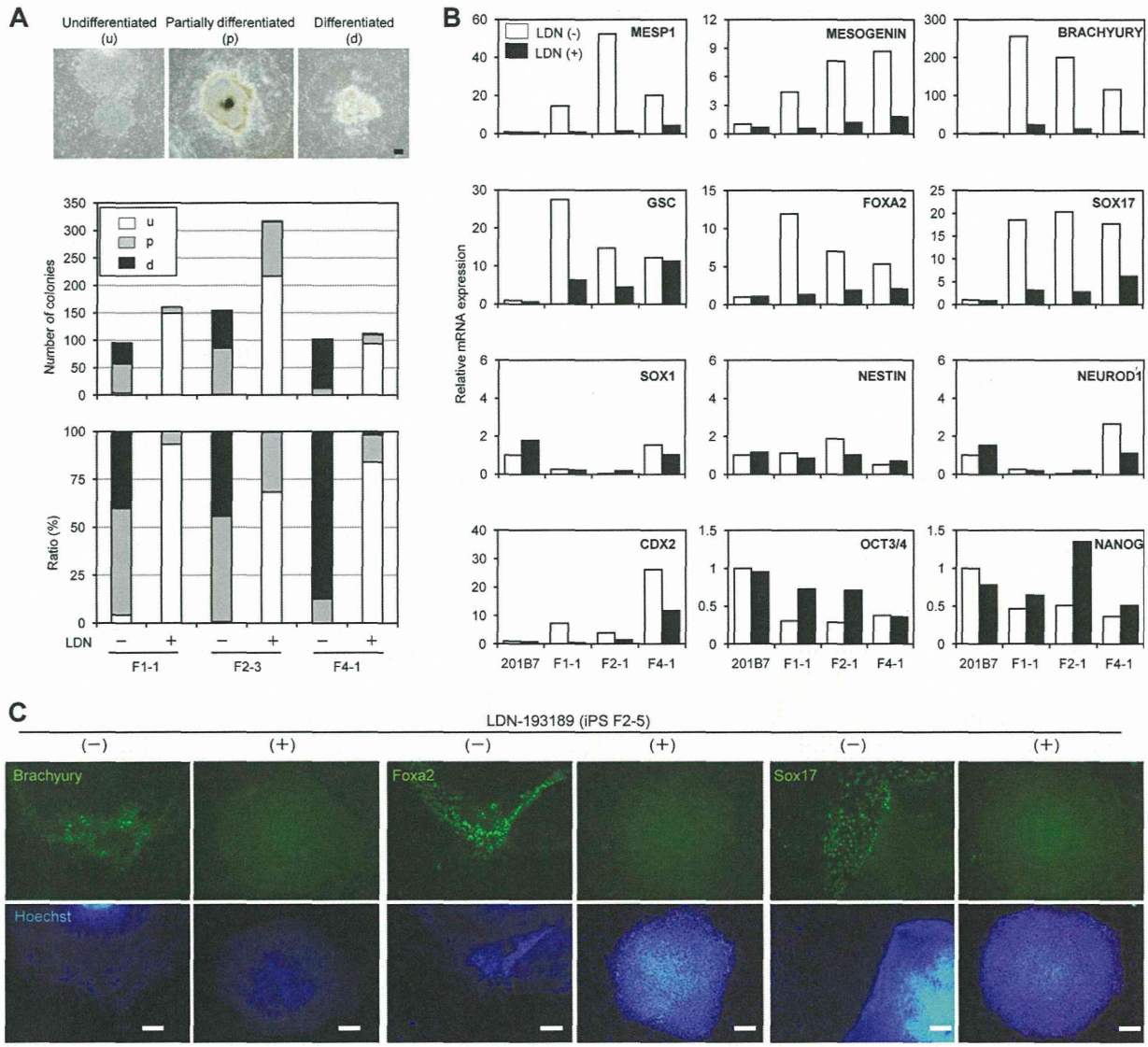


Figure 3. Spontaneous differentiation of fibrodysplasia ossificans progressive FOP-derived iPSCs into mesodermal and endodermal cells. **(A):** Spontaneous differentiation of FOP-derived iPSC lines in the absence of LDN. Undifferentiated (U) indicates an undifferentiated colony, partially differentiated (P) indicates a partially differentiated colony, and differentiated (D) indicates a completely differentiated colony (upper pictures). Number and ratio of the three types of colonies from FOP-derived iPSC lines treated or not treated with inhibitor (center and lower panels). Scale bar = 200 μm. **(B):** Quantitative RT-PCR analysis of the expression of pluripotency- and differentiation-related genes. The data are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and represent expression levels relative to the 201B7 human iPSC line cultured without LDN. **(C):** Immunofluorescence staining of differentiation markers in FOP-derived iPSC lines cultured without LDN. Four iPSC lines were stained and the representative data obtained from the iPSC line F2-5 are shown here. Hoechst staining indicates the nuclear. Scale bars = 200 μm. Abbreviations: iPS, induced pluripotent stem; LDN, LDN-193189.

FOP-derived iPSCs Can Spontaneously Differentiate into Both Mesoderm and Endoderm Lineages Under Conditions for Maintaining iPSCs

The removal of ALK2 inhibitors from FOP-derived iPSC cultures caused the colonies to be disrupted and differentiation to be initiated even under conditions for maintaining iPSCs (Fig. 3A and supporting information Fig. S4). To define the differentiated cell type, the expression of a set of differentiation markers in control and FOP-derived iPSC lines was examined (Fig. 3B, 3C). We observed elevated expression of both mesodermal (MESP1, MESOGENIN, and BRACHYURY) and endodermal (SOX17 and FOXA2) markers in iPSC lines cultured in the absence of the ALK2 inhibitor [29, 30]. CDX2, a marker of trophectoderm [31], was also upregulated in FOP-derived iPSC lines cultured without LDN. In contrast, the expression levels of neuroectodermal markers, such as SOX1, NESTIN, and NEUROD1 [32], did not change, even in the absence of the inhibitor (Fig. 3B). The iPSC lines cultured in the absence of LDN expressed pluripotency markers such as OCT3/4 [33] and NANOG [34, 35] at a lower level than those in the presence of LDN (Fig. 3B). Immunostaining analysis also confirmed the elevated expression of mesodermal and endodermal markers (Fig. 3C). The expression patterns of these markers indicated that FOP-derived iPSCs tend to spontaneously differentiate into both mesodermal and endodermal cells rather than into ectodermal cells, despite being culturing under iPSC maintaining conditions.

Characterization of FOP-Derived iPSCs

We used microarray analysis to further characterize the FOP-derived iPSC lines. The patterns of global gene expression of three FOP-derived iPSC lines treated with LDN were different to those of cells not treated with LDN (Fig. 4A). We confirmed that the expression levels of mesodermal and endodermal markers were higher in iPSCs not treated with LDN than in those treated with LDN, whereas expression levels of

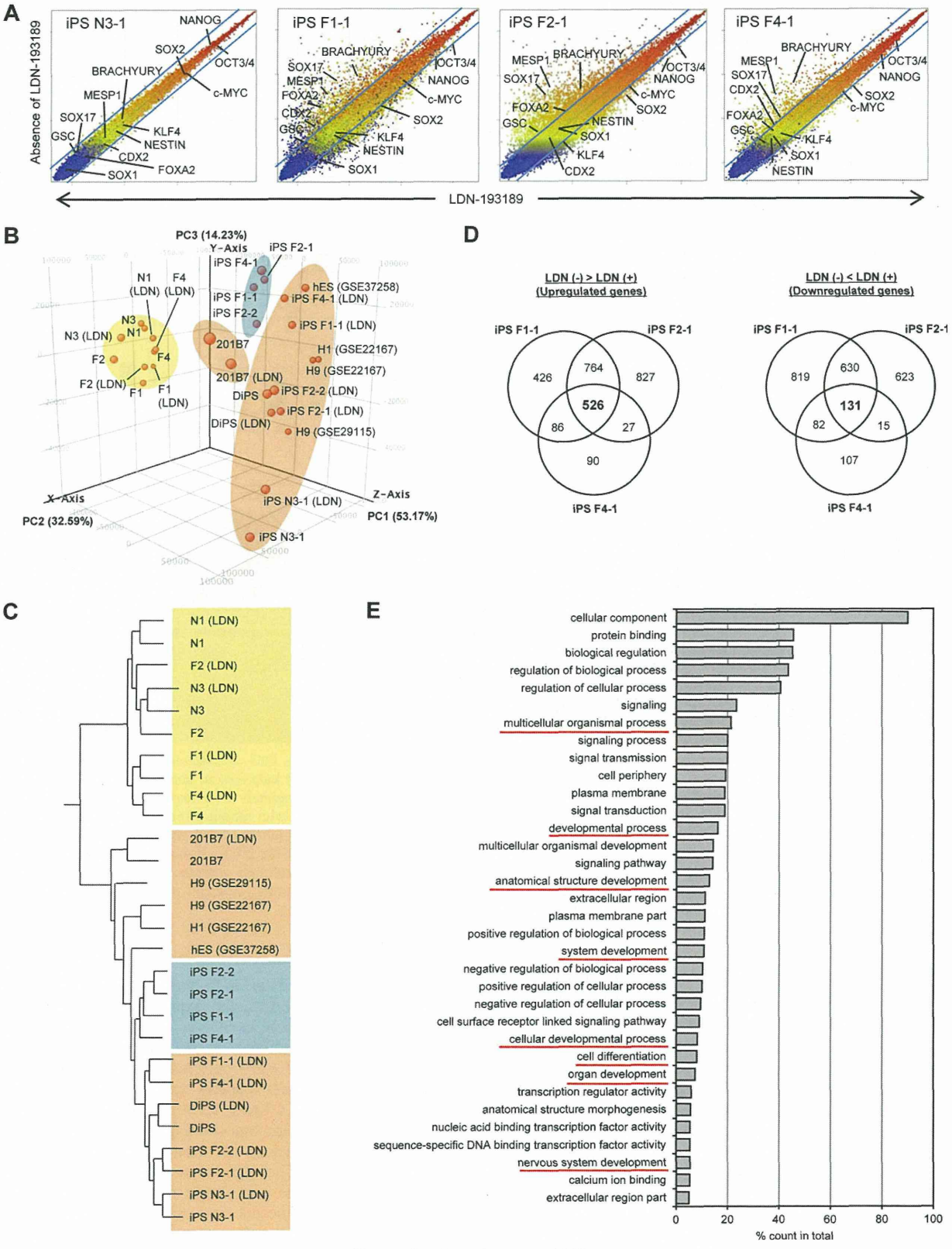


Figure 4. (legend on page 2443).