Recapitulation of pro-inflammatory signature of monocytes with ACVR1 mutation using FOP patient-derived iPSCs.

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Research Article

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Title

Recapitulation of pro-inflammatory signature of monocytes with ACVR1 mutation using FOP patient-derived iPSCs.

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Abstract

Background

Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disease characterized by progressive heterotopic ossification (HO) in soft tissues due to a heterozygous mutation of the ACVR1A/ALK2 gene (FOP-ACVR1A), which erroneously transduces the BMP signal by Activin-A. Although inflammation is known to trigger HO in FOP, the role of FOP-ACVR1A on inflammatory cells remains to be elucidated.

Results

We generated immortalized monocytic cell lines from FOP-iPSCs (FOP-ML) and mutation rescued iPSCs (resFOP-ML). Cell morphology was evaluated during the monocyte induction and after immortalization. Fluorescence-activated cell sorting (FACS) was performed to evaluate the cell surface markers, CD14 and CD16, on MLs. MLs were stimulated by lipopolysaccharide (LPS) or Activin-A and the gene expression was evaluated by quantitative PCR (qPCR) and microarray analysis. Histological analysis was performed for HO tissue obtained from wild type mice and FOP-ACVR1 mice which conditionally express human mutant ACVR1 gene by doxycycline (Dox) administration. Without any stimulation, FOP-ML showed the pro-inflammatory signature of CD16+ monocytes with upregulation of INHBA gene, and treatment of resFOP-ML with Activin-A induced the expression profile mimicking those of FOP-ML at baseline.

Treatment of FOP-ML with Activin-A further induced the inflammatory profile with up-regulation of inflammation-associated genes, some, but not all, of which were suppressed by corticosteroid. Experiments using an inhibitor for TGFβ or BMP signal demonstrated that Activin-A-induced genes such as *CD16* and *CCL7* were regulated by both signals, indicating Activin-A transduced dual signals in FOP-ML. Comparison with resFOP-ML identified several down-regulated genes in FOP-ML including *LYVE-1*, which is known to suppress matrix-formation in vivo. Down-regulation of LYVE-1 in HO tissues was confirmed in FOP model mice, verifying the significance of in vitro experiments.

Conclusion

These results indicate that FOP-ML faithfully recapitulated the phenotype of primary monocytes of FOP and the combination with resFOP-ML is a useful tool to investigate molecular events at the initial inflammation stage of HO in FOP.

Keyword

Fibrodysplasia ossificans progressive (FOP), Monocyte, Inflammation, induced Pluripotent Stem Cell (iPSC), Activin-A, Bone Morphogenic Protein (BMP)

Introduction

Fibrodysplasia ossificans progressiva (FOP) is an extremely rare genetic condition

64 characterized by the systemic and progressive development of mature bone tissues in
65 soft tissues such as skeletal muscles, tendons, and ligaments (heterotopic ossification,
66 HO)[1]. The disease-causing gene is *ACVR1A/ALK2* (hereafter *ACVR1A*) gene, which
67 encodes a type I BMP receptor [2], and more than 95% of patients carry an identical
68 mutation, R206H [3]. In most cases, HO was initiated by an episode of painful swelling
69 (flare-up), and bone tissues were formed at the flare-up site several weeks or months
70 after the episode. Repetitious flare-up episodes gradually spread HO in the trunk and
71 extremities to cause a serious inhibition of daily activity [4]. This stepwise exaggeration
72 of the disease suggested that factors transducing the BMP signal via mutant ACVR1A at
73 the flare-up are key to inducing the HO. Histological findings by archival biopsy samples
74 demonstrated the sequential events of HO in FOP [5]. In the earliest stage, mononuclear
75 cells showing the features of mast cells and macrophages infiltrated at the flare-up sites,
76 which was followed by the proliferation of myofibroblasts, the formation of chondroid
77 tissues, and final bone formation. Each step contributes to HO, but the transition from
78 myofibroblasts to chondrocytes is critical. To recapitulate this process *in vitro*, we have
79 established induced pluripotent stem cells (iPSCs) from the somatic cells of FOP patients
80 (FOP-iPSCs) and also mutation-corrected FOP-iPSCs (resFOP-iPSCs) [5,6]. From
81 these iPSCs, mesenchymal stromal cells (MSCs) were induced (FOP-iMSCs and
82 resFOP-MSCs), and factors to induce chondrogenic differentiation selectively in FOP-

iMSCs were searched. We found that Activin-A, which physiologically transduces the TGFβ signal via a receptor complex with ACVR1B/ALK4, erroneously transduces the BMP signal via mutant ACVR1A to induce HO formation [7]. Identical results were reported by another group using transgenic mice harboring human mutant ACVR1A [8]. This pivotal finding describes the molecular mechanism of FOP and provides a new strategy to treat this intractable disease, such as blocking Activin-A with a neutralizing antibody, inhibiting the Activin-A signal by a mutant-specific kinase inhibitor, and inhibiting the downstream signal by mTOR inhibitors [8-10].

Although the molecular events after the binding of Activin-A to mutant ACVR1A on precursor cells have been gradually disclosed, those in the initial inflammation stage are still equivocal. Activin-A is known to be involved in inflammation [11], but its role on monocytes with FOP-ACVR1 is not yet clear. Nearly half of patients experienced the formation of new HO without a clear episode of flare-up [4], suggesting an abnormal response to inflammatory signals in FOP patients. The importance of the initial step was demonstrated *in vivo* using the genetic or chemical inhibition of mast cells and macrophages, and the depletion of these cells significantly inhibited the HO formation in an FOP mouse model [12]. A comprehensive immunophenotype analysis of FOP patient monocytes identified several surface markers as up-regulated [13]. Specifically, an increase of $CD16^{+}$ cells and the involvement of the p38-MAPK pathway without activating

the canonical signal pathway using the SMAD1/5/9 axis were observed, suggesting the activation of lymphocyte-specific signal pathway [14,15]. Additionally, mutant ACVR1 may have some effects on the biology of inflammatory cells, which may contribute to the development of flare-up and subsequent HO. These data indicated that understanding the effect of mutant ACVR1A/ALK2 is important for clarifying the initial event of FOP. The limited growth potential of monocytes, however, makes it difficult to conduct this analysis in detail. In addition, differences between individuals, such as genetic background and previous history of anti-inflammatory therapy, including oral corticosteroid, may compromise the evaluation of the effect of mutant ACVR1A/ALK2 on monocytes.

To overcome these issues, here we established immortalized monocyte cell lines from FOP- and resFOP-iPSCs (FOP-ML and resFOP-ML) and investigated the effect of FOP-ACVR1A on these cell lines by evaluating gene expression profiles. FOP-ML demonstrated the pro-inflammatory phenotype at baseline with an enhanced expression of *INHBA* gene encoding Activin-A, resulting in the subsequent activation of pro-inflammatory signals. Using a comparative analysis, we identified one molecule involved in the matrix formation, LYVE-1, which is down-regulated in FOP-ML and also in monocytes localized in the HO of FOP mice, suggesting that FOP-ML is a useful model for disclosing the role of FOP-ACVR1A in inflammatory cells.

Materials and Methods

Cell culture

FOP-iPSCs used in this study were established from a FOP patient harboring R206H heterozygous mutation in ACVR1 [16], and mutation-corrected resFOP-iPSCs were generated by BAC-based homologous recombination [6]. iPSCs were maintained in StemFit AK02N (Ajinomot) on iMatrix 511 silk (Nippi)-coated dishes.

Monocytes were induced from iPSCs by a previously described method with some modification [17] , and then immortalized using lentivirus vectors containing *BMI1*, *cMYC*, and *MDM2* genes in the presence of polybrene (Sigma) [18,19]. Immortalized monocyte cell lines (ML) were maintained in StemPro-34 (Gibco) supplemented with 2 mM L-glutamine (Gibco), 50 ng/mL recombinant human macrophage colony stimulating factor (M-CSF) (R&D Systems), and 50 ng/mL recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) (R&D Systems) [20]. $CD14^+$ ML were collected by magnetic-activated cell sorting (MACS) using anti-human CD14 MicroBeads (Miltenyi Biotec) every time before using in each experiment, as per the manufacturer's protocol.

Fluorescence-activated cell sorting (FACS)

FACS was performed by AriaII (BD) according to the manufacturer's protocol. The antibodies used in the FACS are listed in Table S1. In all experiments, FACS histograms of isotype controls were similar to those without antibodies; therefore, histograms without

antibodies were used as control populations.

May-Giemsa staining

FOP- and resFOP-ML were seeded onto MAS-GP type A glass slides (Matsunami) and stained with May-Grunwald and Giemsa staining solution (Merck Millipore) in accordance with the manufacturer's instructions.

Immunocytochemical staining

FOP- and resFOP-ML were fixed by 2% paraformaldehyde for 10 minutes and washed with PBS 3 times. 50~100 μL suspensions containing 50,000~100,000 fixed cells were applied directly to the slide (Matsunami), dried at room temperature, and permeabilized with 100% methanol at 4℃ for 10 minutes. Samples were blocked with Blocking One or Blocking One-P (Nacalai Tesque) for 60 minutes and then incubated with anti-CD14, CD16, LYVE-1, or p-Smad5 antibody diluted in Can Get Signal Immunostain Solution B (Toyobo) for 16 to 18 hours at 4°C. Next, the samples were washed 3 times in 0.2% Tween-20 (Sigma-Aldrich) in PBS and incubated with Alexa Fluor 488 conjugated donkey anti-mouse IgG secondary antibody (Abcam) and Alexa Fluor 647 conjugate donkey anti-rabbit IgG secondary antibody (Thermo Fisher Scientific) diluted in Can Get Signal Immunostain Solution B for 1 hour at room temperature. DAPI (10 μg/mL) was used to counterstain nuclei.

RNA isolation and quantitative polymerase chain reaction

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN) with DNase treatment to remove genomic DNA. Total RNA (0.3 μg) was reverse transcribed into cDNA with ReverTra Ace (Toyobo) in a total volume of 20 μL. Quantitative PCR (qPCR) was performed with Thunderbird SYBR qPCR Mix (Toyobo) and analyzed with QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). The primers used are listed in Table 1. β-Actin was used for normalization as an endogenous control in all data.

Stimulation and inhibition of signals in FOP- and resFOP-ML

For stimulation experiments, cells were seeded at 100,000 cells per well in a 24-well plate. On the next day, ML were stimulated by 10 μM lipopolysaccharides (LPS) (Sigma-Aldrich) or 100 ng/mL Activin-A (R&D Systems) with or without 1 μM Dexamethasone (Wako). ML were collected for RNA extraction or immunostaining 4, 12, or 24 hours after the reagent stimulation. For the inhibition experiments, the cells were stimulated with Activin-A and simultaneously treated with a TGFβ inhibitor (SB431542) or BMP inhibitor (DMH1) for 24 hours. The RNAs were then analyzed as described above.

Microarray analysis

RNA was extracted from FOP- and resFOP-ML stimulated with 10 ng/mL LPS or 100 ng/mL Activin-A for 12 hours and from ML without stimulation as a control (n=3, biological replicates). After the RNA quality was confirmed by the RNA 6000 Nano Kit (Agilent Technologies), all RNA samples were processed using the Ambion WT Expression Kit

(Life Technologies), the GeneChip WT Terminal Labeling and Controls Kit, and the GeneChip Hybridization Wash and Stain Kit (Affymetrix) according to the manufacturer's protocol. Raw CEL files were imported into GeneSpring GX 14.9 software (Agilent Technologies), and the expressions were calculated using the RMA16 algorithm. Heatmaps, principal component analysis (PCA), and Venn diagrams were generated using GeneSpring GX14.9 software. Upstream analysis was performed using Ingenuity Pathway Analysis (IPA) (QIAGEN). Array data were deposited in the NCBI's Gene Expression Omnibus (GEO) database (GEO GSE 183525).

Induction of HO in FOP mice

The establishment of FOP-ACVR1 conditional transgenic mice (FOP mice) was reported previously, in which the expression of mutant ACVR1 gene is induced by the administration of doxycycline[10]. Female mice 13- to 17-weeks old were used in the experiments, and HO was induced by a pinch injury as previously described[21]. From 7 days before the pinch injury, mice were fed water supplemented with 2 mg/mL doxycycline and 10 mg/mL sucrose, and the left gastrocnemius muscle was pinched using tissue forceps for 5 s. Tissue samples were collected 14 days after the pinch injury from mice euthanized by carbon dioxide (CO_2). The age and body weight at the start point of each experiment were matched between groups.

Induction of HO in wild-type mice

HO was induced in wild-type (WT) mice by collagenase injection into the Achilles tendon as previously described [22]. Eight-week-old male C57BL/6NJcl mice (Clea Japan) were used, and 20 μL of 1% collagenase (FUJIFILM Wako)/PBS was injected into their Achilles tendon under anesthesia using a mixture of medetomidine, midazolam, and butorphanol. Six weeks after the injection, tissue samples were collected after euthanization by CO_2.

Histological analysis

Collected tissue samples from mice were fixed with 4% paraformaldehyde for 48 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and Safranin O. Mice tissue sections were also processed as above with F4/80 (Abcam) and LYVE-1 antibody (Abcam) after deparaffinization using Clear Plus (Falma) without permeabilization. All samples were observed with a BZ- X810 (Keyence).

Statistics

Statistical analysis was performed using JMP Pro 15 (SAS Institute Inc). Statistical significance was evaluated by two-way ANOVA followed by a Tukey-Kramer multiple comparison test or Dunnett's multiple comparison test. P values less than 0.05 were considered statistically significant. All studies were performed and analyzed with biological replicates.

Results

Establishment of FOP- and resFOP-ML

Monocyte cell lines were indued from FOP-iPSCs and resFOP-iPSCs. Cellular and colony morphology during the monocyte induction showed no difference between FOP- and resFOP-clones (Figure 1A). After 18-21 days of monocyte induction, floating cells were collected, and $CD14^+$ monocytes were sorted using MACS (Figure 1B) and immortalized using lentivirus vectors encoding *BMI1*, *cMYC*, and *MDM2* genes[18,19]. Proliferating $CD14^+$ monocyte-derived cells were obtained from both FOP- and resFOP-iPSCs (FOP-ML and resFOP-ML) (Figure 1C). The morphology of each was compatible with those of primary monocytes, and there was no clear difference between FOP-ML and resFOP-ML (Figure 1D). The expression of CD14 in FOP-ML and resFOP-ML was further confirmed by FACS and showed an almost equal profile (Figure 1E), whereas the population of cells expressing CD16 seemed to be larger in FOP-ML than in resFOP-ML (Figure 1F).

Characteristics of FOP-ML

To evaluate the effect of mutant ACVR1 on monocytes, we compared the characteristics of FOP-ML and resFOP-ML at baseline and under the stimulation of LPS or Activin-A. The phosphorylation of Smad5, a downstream marker of BMP signaling,

was upregulated in FOP-ML at baseline and enhanced by Activin-A, but not in resFOP-ML (Figure 2A). This result indicates that mutant ACVR1 specific signaling was transduced in FOP-ML. $CD16^+$ monocytes are regarded as a pro-inflammatory subpopulation [15]. Immunocytochemical staining showed that the proportion of $CD16^+$ cells was higher in FOP-ML than resFOP-ML at baseline (Figure 2A). LPS stimulation failed to increase the proportion of $CD16^+$ cells in either group, but Activin-A stimulation induced $CD16^+$ cells in both groups, although the induction was greater in FOP-ML (Figure 2A). A qPCR analysis of *CD14* and *CD16* genes showed compatible results with those of the immunostaining. The baseline expression of *CD14* showed no difference between resFOP- and FOP-ML, and LPS increased the expression of *CD14* in resFOP-ML but not in FOP-ML, whereas Activin-A induced no changes in either cell lines (Figure 2B). In the case of *CD16*, however, its baseline expression in FOP-ML was higher than in resFOP-ML, and Activin-A increased the expression in both cell lines over time (Figure 2C). These results suggested that FOP-ML may receive an Activin-A signal at baseline. In agreement with this hypothesis, the expression of *INHBA* gene, which encodes the alpha subunit of Activin-A, was much higher in FOP-ML than in resFOP-ML at baseline and continued to be highly expressed during treatment with Activin-A (Figure 2D). The expression of *FOP-ACVR1* gene showed no change during the culture period (Figure 2E).

Gene expression profiles of resFOP-ML and FOP-ML before and after stimulation

To investigate the effect of mutant ACVR1 on FOP-ML in detail, the entire gene expression profile was compared between FOP- and resFOP-ML by microarray. PCA demonstrated a clear difference between the two groups at baseline (Figure 3A). After stimulation with LPS, a significant shift (Figure 3A, indicated by the green arrows) was observed in both FOP- and resFOP-ML, showing movement with a similar direction and distance in the PC1 or PC2 component. The shift after Activin-A stimulation, however, showed a significant difference between the two (Figure 3A, indicated by the black arrows). resFOP-ML showed only little shift after the stimulation and moved toward FOP-ML at baseline. On the other hand, FOP-ML showed a significant shift in the PC1 component and approached the position after LPS stimulation.

The transition of the gene profiles during the 24-h treatment was compared by clustering using the expression profile of genes up-regulated by LPS in both resFOP- and FOP-ML, such as *IL1B* and *IL6* (Figure S1). As for LPS-treated samples, both FOP- and resFOP-ML were found in the same cluster, and a heatmap showed a similar intensity of representative genes. On the other hand, FOP-ML and resFOP-ML created cell-type-specific clusters after treatment with Activin-A, and the intensity of cluster-defined genes was significantly different. These results suggested the FOP-ML-specific

features are Activin-A dependent.

Identification of Activin-A-induced features in FOP-ML

Volcano plots visualized up- and down-regulated genes in FOP-ML when compared with resFOP-ML at baseline (Figure 3B) or after Activin-A stimulation (Figure 3D). *IL1B*, *TCF4*, and *MMP12* genes were found among the up-regulated genes at baseline. TCF4 is a transcription factor that transduces the Wnt/β-catenin signal and is reported to be expressed in $CD16^{+}$ pro-inflammatory monocytes [23]. MMP12, a member of the matrix protease family, is secreted by pro-inflammatory macrophages [24] and regulated by NFκB and β-catenin [25]. IL1β is a pro-inflammatory cytokine secreted by activated monocytes and macrophages and plays a key role in inflammatory responses [26]. One of the signals regulating the expression of *IL1B* is the non-canonical BMP signal in association with PU.1 [27], which has been shown to be expressed in pro-inflammatory monocytes[28]. A number of metallothionein genes were found among the down-regulated genes; these genes have been shown to inhibit the differentiation of monocytes [29] and are negatively regulated by the TGFβ signal via PU.1 [30,31]. IPA identified several signal pathways that promote monocyte activity as upstream pathways in FOP-ML (Figure 3C) [28], indicating that FOP-ML at baseline are already activated.

A volcano plot after 12 h of Activin-A stimulation demonstrated newly up-regulated

genes, such as *CCL7* and *CCL13* (Figure 3D). CCL7, also known as monocyte chemoattractant protein 3, is a secreted chemokine which directs chemotaxis in monocytes during inflammation [32]. CCL13, also known as monocyte chemoattractant protein 4, is also a monocytic chemokine with chemotactic activity [33]. The IPA-listed upstream regulators showed almost the same signals identified at baseline (Figure 3E), further confirming that FOP-ML received the Activin-A signal at baseline.

The role of Activin A for the up-regulation of these genes was further investigated by comparing resFOP- and FOP-ML (Figure 3F/G). LPS induced the expression of these genes in both cell lines in a time-dependent manner, whereas Activin-A induced these genes earlier and more in FOP-ML than in resFOP-ML, suggesting signals via mutant ACVR1 are involved in the up-regulation of these genes.

TGFβ and BMP signals for the regulation of genes in FOP-ML

Activin-A transduces both TGF and BMP signals in FOP cells [7]. To investigate the signal responsible for the feature of FOP-ML, Activin-A-treated FOP-ML were treated with an inhibitor for the TGFβ (SB) or BMP (DMH1) signal. The expression of *INHBA* was reduced by SB but not by DMH1, indicating that the induction of *INHBA* by Activin-A is mainly via the TGFβ signal pathway (Figure 4A). Neither inhibitor changed the expression of *FOP-ACVR1* (Figure 4B). The expression of *CD14* showed no difference

311 by either inhibitor treatment (Figure 4C). On the other hand, the expression of *CD16*
312 gene induced by Activin-A was inhibited by SB at an earlier time point than by DMH1
313 (Figure 4D). SB also inhibited the gradually increased expression of *IL6* by Activin-A
314 (Figure 4E). In contrast, the expression of *CCL7* gene was suppressed by both inhibitors
315 even at the earlier time point, suggesting the direct involvement of both signals for the
316 regulation of this gene (Figure 4F).
317
318 **Effect of corticosteroid on activated FOP-ML**
319 To investigate whether the induced expression of these genes by Activin-A can be
320 controlled by drugs, Activin-A-treated cells were simultaneously treated with
321 dexamethasone, which is one of several corticosteroids currently used as Class I
322 mediations for FOP patients, especially at the flare-up[34]. The up-regulation of *IL1B*, *IL6*
323 and *CCL7* genes by Activin-A in FOP-ML was inhibited by dexamethasone (Figure
324 5A,B,C), but the expression of *INHBA* was negligibly affected (Figure 5D), suggesting
325 the limited therapeutic effects of corticosteroids for FOP.
326
327 **Identification of target genes regulated by Activin-A in FOP-ML**
328 Using the combination of several Venn diagrams, genes regulated by Activin-A in
329 FOP-ML were searched (Figure 6A/B), and 10 up-regulated (Figure 6C) and 3 down-

330 regulated genes (Figure 6D) were identified. *EIF4B*, *ID3*, and *LTC4S* were among the
331 up-regulated genes. EIF4B (eukaryote initiation factor 4B) is a member of the EIF family,
332 which regulates translation in general and is one of the downstream molecules of the
333 mTOR pathway [35]. The Ras-MAPK pathway was also shown to regulate its expression
334 [36]. Since our previous study showed that FOP-ACVR1 abnormally transduces BMP
335 signaling via the mTOR pathway in response to Activin-A [10], this result demonstrated
336 that the FOP-ACVR1-specific signal is transduced in FOP-ML. LTC4S is an enzyme that
337 converts leukotriene A4 to create leukotriene C4, which is a mediator of anaphylaxis and
338 inflammatory conditions [37], an important molecule in mast cells [38], and regulated by
339 the ERK/NFκB pathway [39]. ID3 is a transcription factor and target gene of BMP, but
340 Activin-A enhanced its expression, and DMH1 and SB suppressed it at earlier time points
341 (Figure 6E).

342 One of the down-regulated genes, *LYVE-1* (Lymphatic vessel endothelial
343 hyaluronan receptor 1), encodes a receptor of hyaluronan [40]. Inhibition experiments
344 indicated that the TGFβ signal is responsible for the suppression of this gene (Figure 6F).

345 **Expression of LYVE-1 was down-regulated in monocytes with FOP-ACVR1 *in vitro***
346 **and *in vivo***

347 Although LYVE-1 was originally expected to be expressed selectively in
348 lymphangitic cells [40], recent data demonstrated its expression in

monocytes/macrophages and its involvement of matrix formation [41]. An immunocytochemical analysis showed the expression of LYVE-1 in resFOP-ML, but hardly in FOP-ML after treatment with Activin-A (Figure 7A). Because the putative function of LYVE-1 is related to matrix formation [42], we further analyzed this molecule *in vivo* using pinch-injury-induced HO tissues from FOP-ACVR1 mice and collagenase-induced HO from WT mice (Figure 7B). H&E and Safranin-O staining showed heterotopic cartilage formation in the Achilles tendon of WT mice, and F4/80 positive monocytes and macrophages were found adjacent to the HO, which was also positive for LYVE-1. On the contrary, F4/80 positive cells adjacent to heterotopic cartilage tissue developed at the injured site of FOP-ACVR1 mice were negative for LYVE-1 (Figure 7B). These *in vivo* results agree with the *in vitro* data, suggesting the usefulness of FOP-ML to identify the pathologic change in monocytes with FOP-ACVR1 and that FOP-ML are a promising tool to find new therapeutic approaches.

Discussion

iPSCs derived from patients with a particular type of hereditary disease (disease-specific iPSCs) have been widely used to investigate the disease-causing mechanisms and develop therapeutic drugs [43]. There are several advantages to disease-specific iPSCs. The induction of target cells from the iPSCs can be repeated and therefore there is no limitation in the number of cells available for the analysis. Additionally, the effect of the genetic background can be avoided by gene editing the iPSCs. Finally, different types of cells from the same iPSC line, in other words from the same patient, can be analyzed if the appropriate induction methods are available. We have been applying this strategy to a number of musculoskeletal diseases and successfully recapitulated the diseases in vitro and identified candidate drugs [44,46]. In the case of FOP, we induced MSCs from FOP- and resFOP-iPSCs (FOP-iMSCs and resFOP-MSCs), investigated the transition from MSCs to chondrocytes and identified Activin-A as a key factor to initiate the process of HO [7]. In the present report, we focused on the initial phase of HO and analyzed the effect of the ACVR1A mutation on monocytes by comparing the gene expressions of FOP-ML and resFOP-ML. Monocytes, macrophages, and mast cells induced from iPSCs have been used to analyze hereditary inflammatory conditions before [19,47]. FOP-ML showed a gene expression profile consistent with the pro-inflammatory status, as if they had been stimulated by inflammatory cytokines such as TNFα or LPS at baseline. These

data agree with those of primary cells collected from FOP patients [13,15]. In this regard, the up-regulation of *INHBA* gene may play a central role in the accelerated inflammatory status of FOP-ML at baseline and after stimulation.

Several inflammatory-related genes, including *IL1B*, *MMP12*, and *TCF4*, were up-regulated at baseline in FOP-ML, while Activin-A stimulation induced the expression of *CCL7* and *CCL13* genes. Experiments using inhibitors indicated some of these genes are directly regulated by TGFβ and/or BMP signals induced by Activin-A, among which the expression of *CCL7* seemed to be regulated by both signals. Previously, we demonstrated that Activin-A induced both TGFβ and BMP signals in FOP-MSCs and that both were indispensable to induce the expression of *ENPP2* gene, which then induces the chondrogenic differentiation of FOP-MSCs via activation of the mTOR complex [10]. *CCL7* may be one of many molecules regulated by both signaling pathways in cells with FOP-ACVR1A. In contrast, the regulation of other genes is not simple. LPS is known to induce *IL1B* gene via the NFkB signal [48]. We found Activin-A induced the expression of *IL1B* in FOP-ML but not in resFOP-ML, suggesting the dual signals induced by Activin-A in FOP-ML may crosstalk with the NFkB signal pathway [49]. Multiple experiments using ligands or inhibitors for each signal are needed to understand the complex crosstalk. Immortalized FOP-ML and resFOP-ML will be suitable for these experiments.

FOP-ML are also a useful tool for the search of therapeutic targets in the initial

401 inflammatory stage of FOP. For example, we identified LTC4S as an Activin-A-regulated
402 molecule through microarray analysis. Although the therapeutic effect of leukotriene
403 inhibitors is limited and the drugs are categorized as class II medication for flare-up [34],
404 our data suggest the prophylactic use of leukotriene inhibitors for suppressing the early
405 event of HO. Dexamethasone, class I medication, suppressed the expression of
406 inflammatory cytokines in FOP-ML, however it did not have an inhibitory effect for the
407 abnormal INHBA expression in FOP-ML. Higher Activin-A production from FOP patient
408 derived M1 macrophages is also reported from a previous report [50]. The treatment that
409 can inhibit higher expression of INHBA in FOP monocytes would be prospective to
410 ameliorate HO formation and it would be found from our FOP-ML via high-throughput
411 screening. The identification of LYVE-1 as a gene down-regulated by Activin-A is an
412 intriguing finding when its function in matrix formation is considered. LYVE-1 is a marker
413 for distinguishing between blood and lymphatic vessels and plays an important role in
414 leukocyte trafficking [40]. Recent data demonstrated that LVYE-1 is also expressed on
415 monocytes/macrophages, which exist not only around the arteries but also in skeletal
416 muscle [41]. LYVE-1 on macrophages activates MMP-9 by engaging hyaluronic acid
417 and maintains the elasticity of the arterial wall by the MMP-9-dependent degradation of
418 collagen [20]. In the present report, for the first time, we demonstrated that
419 monocytes/macrophages localizing adjacent to HO tissues express LYVE-1 in the

collagenase-induced HO model. Although the significance of this expression is not yet clear, the proposed function of LYVE-1 for the degradation of collagens may contribute to the limited HO formation in this model. On the other hand, almost no expression of LYVE-1 was found in monocytes/macrophages localizing adjacent to HO tissues in FOP mice. Considering the suppression of LYVE-1 expression by Activin-A and the elevated expression of *INHBA* gene in FOP-ML, monocytes/macrophages in FOP mice may contribute to uncontrolled HO formation by the loss of LYVE-1 expression, which results in the failure of collagen degradation. Although further experiments are necessary, these data suggest LYVE-1 as a new target for FOP therapy.

Conclusion

In this study, we established immortalized monocyte cell lines from FOP- and resFOP-iPSCs (FOP-ML and resFOP-ML) and demonstrated the pro-inflammatory status of the former. Most features of FOP-ML are compatible with those observed in primary monocytes collected from FOP patients, validating the use of FOP-ML as an unlimited cellular source for FOP study.

Additional files

Additional file 1: Fig S1. Hierarchical clustering of samples treated with LPS or Activin-A

Additional file 2: Table S1. Primers for qRT-PCR

Abbreviations

FOP: Fibrodysplasia ossificans progressiva; HO: Heterotopic ossification; iPSCs: induced pluripotent stem cells; MSC: mesenchymal stromal cell; H&E: Hematoxylin and eosin

Declarations

Ethics approval and consent to participate

All experimental protocols dealing with human subjects were approved by the ethics committee of the Department of Medicine and Graduate School of Medicine of Kyoto University. Written informed consent was provided by each donor. All animal experiments were approved by the institutional animal committee of Kyoto University.

Consent for publication

Written informed consent for publication was provided by each donor.

Availability of data and materials

The data used during this study are available from the corresponding author on

458 reasonable request.

459

460 **Competing interests**

461 The authors declare no conflicts of interest.

462

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472

473 **Authors' contributions**

474 H.M and J.T. designed the research and wrote the manuscript. Y.J. designed and
475 provided some in vitro experiment data. M.N, S.N, and T.K. helped with data collection.
476 S.K. provided in vivo study material and advised on the project. H.Y. advised on the

project. A.N, and M.K.S provided the study materials and advised on the project. S.M. advised on the project.

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FIGURE LEGENS

Fig 1. Establishment of immortalized monocytic lineage cell lines (MLs) from FOP- and resFOP-iPSCs**. A**, Morphology of colonies during monocyte induction from iPSCs. Representative phase contrast images at indicated time point during step-wise induction stages shown in the top. Scale bars = 500μm. B, Representative phase contrast images of monocytes induced from each iPSC. Scale bars = 500μm. **C**, Representative phase contrast images of each ML. Scale bars = 100μm. **D**, Representative morphology of each ML stained by May-Giemsa staining. Scale bars = 10μm. **E**-**F**, Flow cytometric analyses of resFOP-ML and FOP-ML for the expression of CD14 (E) and CD16 (F).

Fig 2. Characteristics of FOP- and resFOP-MLs with and without stimulations. FOP- and resFOP-MLs were treated with either LPS (10ng/ml) or Activin-A (100ng/ml) for 24hr. **A**, Immunostaining of CD14, CD16, and p-Smad5. Cells were stained before and after 24hr treatment of each chemical. Scale bar = 50μm. **B**-**E**, Time course analyses of

mRNA expression stimulated by LPS or Activin-A. RNAs were extracted at each time point and assessed for the expression of *CD14* (**B**), *CD16* (**C**), *INHBA* (**D**), and *FOP-ACVR1* (**E**) genes by quantitative reverse transcriptase PCR (qPCR). The expression level of each point was shown as a value relative to those of resFOP-ML before treatment. The results were obtained in four biologically independent experiments. The error bars indicate standard deviation. Tukey-Kramer test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fig 3. Gene expression profiles of FOP- and resFOP-ML before and after stimulation. **A**, Principle component analyses. FOP- and resFOP-MLs were treated with LPS (10ng/ml) or Activin-A (100ng/ml) for 24hr and RNAs were extracted at each time point from three biologically independent experiments and processed for microarray analyses. Green and blue circle enclosed samples treated with LPS or Activin-A, respectively. Green and blue arrow indicated the migration from control sample (without treatment). **B**-**E**, Volcano plot and the list of upstream regulators. Expression level of each gene were compared between FOP-ML and resFOP-ML at baseline (**B**) and after the stimulation of Activin-A for 12hr (**D**). Representative up-regulated (red) and down-regulated (blue) genes were shown (cutoff: fold change greater than 1.2; p value less than 0.05). The list of upstream regulators identified by IPA at each comparison were

659 also shown with Z-score (**C** and **E**). **F**-**G**, Time course analyses of mRNA expression
660 stimulated by LPS or Activin-A. RNAs were extracted at each time point and assessed
661 for the expression of *IIL1B* (**F**), and *CCL7*(**G**) genes by quantitative reverse
662 transcriptase PCR (qPCR). The expression level of each point was shown as a value
663 relative to those of resFOP-ML before treatment. The results were obtained in four
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665 ***p<0.001, ****p<0.0001.
666
667 **Fig 4.** Effect of TGFβ or BMP signal inhibitors for the expression of Activin-A-induced
668 genes in FOP-ML**.** FOP-MLs were treated simultaneously with Activin-A (100ng/ml) and
669 SB431542 (SB) (5μM) or DMH1 (5μM). RNAs were extracted at each time point and
670 assessed for the expression of *INHBA* (**A**), *FOP-ACVR1* (**B**), *CD14* (**C**), *CD16* (**D**),
671 *IL6*(**E**), or *CCL7* (**F**) genes by quantitative reverse transcriptase PCR (qPCR). The
672 expression level of each point was shown as a value relative to those of FOP-ML
673 before treatment. The samples were collected from three biologically independent
674 experiments. Two-way ANOVA followed by Dunnett's multiple comparison test (vs
675 DMSO group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
676
677 **Fig 5.** Effect of corticosteroid for gene expression induced by Activin-A. Cells were

678 treated with Activin-A (100ng/ml) and dexamethasone (1μM) for 12hr and the
679 expression of *IL1B*(**A**), *IL6*(**A**), *CCL7*(**A**), or *INHBA*(**A**) were analysed by qPCR. The
680 expression level of each point was shown as a value relative to those of resFOP-ML
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684 **Fig 6.** Identification of target genes regulated by Activin-A in FOP-ML. **A**, Hierarchical
685 clustering of samples treated with LPA or Activin-A. Genes up- or down-regulated in
686 both FOP-ML and resFOP-ML after the stimulation of LPS were selected (shown at the
687 bottom of figure) and used for clustering. The expression level of each point was
688 visualized as a value relative to the mean value of each gene in all samples. Minimum
689 (blue) represents downregulation and maximum (red) represents the degree of
690 upregulation. **B** and **D**, Venn diagrams showing the overlap of DEGs between different
691 comparison groups for up-regulated (**A**) or down-regulated (**D**) genes. **C** and **E**, The list
692 of up- (**C**) and down-regulated (**E**) genes. **F** and **G**, Effect of TGFβ or BMP signal
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701
702 **Fig 7.** Identification of LYVE-1 as a down-regulated gene *in vitro* and *in vivo*. **A**,
703 Expression of LYVE-1 in resFOP-ML and FOP-ML. Cells were treated with or without
704 Activin-A for 12hr, and stained by anti-CD14, anti-LYVE-1 antibodies and DAPI. Scale
705 bar = 50μm. **B**, Expression of LYVE-1 in monocytes localized in heterotopic ossification
706 lesions. Tissues were taken from collagenase injected sites (WT) or pinch-injured sites
707 (FOP) and stained with H&E or Safranin-O. Expression of LYVE-1 in
708 monocyte/macrophage localized adjacent to heterotopic ossification site (indicated by
709 white rectangle) was analyzed by anti-LYVE-1 antibody along with anti-F4/80 antibody
710 and DAPI. The samples were collected from three biologically independent
711 experiments. Scale bar = 250μm.

Figures

Figure 1

Establishment of immortalized monocytic lineage cell lines (MLs) from FOP- and resFOP-iPSCs. A, Morphology of colonies during monocyte induction from iPSCs. Representative phase contrast images at indicated time point during step-wise induction stages shown in the top. Scale bars = 500μm. B, Representative phase contrast images of monocytes induced from each iPSC. Scale bars = 500μm. C, Representative phase contrast images of each ML. Scale bars = 100μm. D, Representative morphology of each ML stained by May-Giemsa staining. Scale bars = 10μm. E-F, Flow cytometric analyses of resFOP-ML and FOP-ML for the expression of 634 CD14 (E) and CD16 (F).

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Effect of TGFβ or BMP signal inhibitors for the expression of Activin-A-induced genes in FOP-ML. FOP-MLs were treated simultaneously with Activin-A (100ng/ml) and SB431542 (SB) (5μM) or DMH1 (5μM). RNAs were extracted at each time point and assessed for the expression of INHBA (A), FOP-ACVR1 (B), CD14 (C), CD16 (D), IL6(E), or CCL7 (F) genes by quantitative reverse transcriptase PCR (qPCR). The expression level of each point was shown as a value relative to those of FOP-ML before treatment. The samples were collected from three biologically independent experiments. Two-way ANOVA followed by Dunnett's multiple comparison test (vs DMSO group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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Effect of corticosteroid for gene expression induced by Activin-A. Cells were treated with Activin-A (100ng/ml) and dexamethasone (1μM) for 12hr and the expression of IL1B(A), IL6(A), CCL7(A), or INHBA(A) were analysed by qPCR. The expression level of each point was shown as a value relative to those of resFOP-ML before treatment. The data was obtained from four biologically independent experiments. Tukey-Kramer test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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Figure 7

Identification of LYVE-1 as a down-regulated gene in vitro and in vivo. A, Expression of LYVE-1 in resFOP-ML and FOP-ML. Cells were treated with or without Activin-A for 12hr, and stained by anti-CD14, anti-LYVE-1 antibodies and DAPI. Scale bar = 50μm. B, Expression of LYVE-1 in monocytes localized in heterotopic ossification lesions. Tissues were taken from collagenase injected sites (WT) or pinch-injured sites (FOP) and stained with H&E or Safranin-O. Expression of LYVE-1 in monocyte/macrophage localized adjacent to heterotopic ossification site (indicated by white rectangle) was analyzed by anti-LYVE-1 antibody along with anti-F4/80 antibody and DAPI. The samples were collected from three biologically independent experiments. Scale bar = 250μm.

Supplementary Files

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