Activin-A enhances mTOR signaling to promote aberrant chondrogenesis in fibrodysplasia ossificans progressiva

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Introduction

Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disease characterized by extraskeletal bone formation in soft tissue, where bone normally does not exist. Ectopic bones are formed through endochondral ossification, a process in which bone tissue replaces mature cartilage (1–7). Patients with classic FOP have a 617G>A (R206H) point mutation in the intracellular glycosyl-phosphatidylinositol (GPI)-anchor domain of ACVR1, a type I receptor for BMPs. Although mutated ACVR1 (FOP-ACVR1) has been shown to render hyperactivity in BMP signaling, we and others have uncovered a mechanism by which FOP-ACVR1 mistransduces BMP signaling in response to Activin-A, a molecule that normally transduces TGF-β signaling. Although Activin-A evokes enhanced chondrogenesis in vitro and heterotopic ossification (HO) in vivo, the underlying mechanisms have yet to be revealed. To this end, we developed a high-throughput screening (HTS) system using FOP patient–derived induced pluripotent stem cells (FOP-iPSCs) to identify pivotal pathways in enhanced chondrogenesis that are initiated by Activin-A. In a screen of 6,809 small-molecule compounds, we identified mTOR signaling as a critical pathway for the aberrant chondrogenesis of mesenchymal stromal cells derived from FOP-iPSCs (FOP-iMSCs). Two different HO mouse models, an FOP model mouse expressing FOP-ACVR1 and an FOP-iPSC–based HO model mouse, revealed critical roles for mTOR signaling in vivo. Moreover, we identified ENPP2, an enzyme that generates lysophosphatidic acid, as a linker of FOP-ACVR1 and mTOR signaling in chondrogenesis. These results uncovered the crucial role of the Activin-A/FOP-ACVR1/ENPP2/mTOR axis in FOP pathogenesis.
To this end, we established an iPSC-based high-throughput screening (HTS) system using FOP-iMSCs and Activin-A–induced enhanced chondrogenesis (38, 44, 45) and used it to screen our HTS library, which contains approximately 7,000 small-molecule compounds. This screening led us to identify mTOR signaling as a critical downstream pathway of FOP-ACVR1 in enhanced chondrogenesis, an essential step of HO in the pathology of FOP. Rapamycin, a commercially available drug and commonly used mTOR inhibitor (46–48), showed potent therapeutic effects on HO in 2 different in vivo models triggered by Activin-A: FOP model mice expressing FOP-ACVR1 and an FOP-iPSC–based HO model, in which ectopic bones derived from FOP patient–derived cells are formed in mice. We also identified ENPP2 (also known as autotaxin) as an upstream molecule that positively regulates the mTOR activity triggered by Activin-A. These data uncovered a molecular basis for the HO induced in patients with FOP.

**Results**

**Development of an HTS system focused on enhanced chondrogenesis of FOP-iMSCs.** Ectopic chondrogenesis is a critical step of endochondral heterotopic ossification in FOP patients (1–6), and enhanced chondrogenesis of FOP-iMSCs was observed when cells were stimulated with Activin-A in vitro and in vivo (38). Therefore, to monitor the degree of enhanced chondrogenesis, an HTS system was established using FOP-iMSCs stably harboring luciferase following 5-repeats Aggrecan enhancers, a well-established chondrogenesis marker (7, 49) (FOP-5×A-Luc-iMSCs; Figure 1A). As expected, the induction of chondrogenesis with Activin-A stimulation in a 384-well plate increased luciferase activity in FOP-5×A-Luc-iMSCs (Figure 1B). Next, we confirmed the expression level of Aggrecan mRNA (ACAN), the glycosaminoglycan (GAG) production to DNA ratio (GAG/DNA), which represents the extracellular matrix amount of chondrocytes, and Alcian blue staining (acidic polysaccharides such as GAG in chondrocytes) in a 2D chondrogenesis induction (2DCI) assay using FOP-5×A-Luc-iMSCs (Figure 1, C–E). Consistent with the luciferase assay (5×A-Luc assay), Activin-A stimulation increased these values in the 2DCI assay. Therefore, we concluded that the 5×A-Luc assay could be used to monitor the degree of enhanced chondrogenesis in HTS format (384 wells). Inhibition of either BMP signaling or TGF-β signaling with a specific inhibitor (DMH1 or SB-431542, respectively) showed reduced luciferase activity, indicating the pivotal roles of both signaling pathways in chondrogenesis. These data confirmed that the HTS assay we developed can evaluate the enhanced chondrogenesis of FOP-iMSCs stimulated by Activin-A.

**HTS and follow-up screens of FOP-iPSCs highlight mTOR signaling as a candidate pathway for modulating enhanced chondrogenesis in FOP.** Using this luciferase-based HTS system (5×A-Luc assay), we performed a first screening (n = 1; test compounds = 0.01, 0.1, and 1 μM) against our HTS library, which contains approximately 7,000 small-molecule compounds, most of which are bioactive and annotated (Figure 2A, Table 1, and Supplemental Figure 1, A and B; supplemental material available online with this article; https://doi.org/10.1172/JCI93521DS1). The scatter plot distribution (Figure 2B), histogram of raw data (Figure 2C), Z-factor, and signal-to-background (S/B) ratio (Supplemental Figure 1, C and D) confirmed the validity of the HTS campaign. From the first screening, we obtained 549 hit compounds that showed more than 40% inhibition of luciferase activity against DMSO control cells stimulated with Activin-A. RARγ agonists and dorsomorphin were identified as positive chemicals, indicating the accuracy of our HTS system. A second screening was performed against these 549 compounds (n = 2; test compounds = 0.1, 0.1, and 1 μM), and we identified 76 hit compounds that showed an IC₅₀ of less than 1 μM in the 5×A-Luc assay and low cytotoxicity in the alamarBlue assay (inhibition of <20% at any dose) (Supplemental Figures 2 and 3 and Table 2). In the classification of these 76 hit compounds, we found several reported compounds, such as corticosteroids (22, 35), RAR agonists (28, 35–37), and ALKS inhibitor (38), that

**Table 1. Source and number of compounds in the HTS library**

<table>
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<th>Library name</th>
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<td>Sigma-Aldrich</td>
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<td>TimTec</td>
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<td>MicroSource Discovery Systems</td>
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<td>Total</td>
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<td>6,809</td>
</tr>
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**Table 2. Classification of 76 hit compounds through the second screening (related to Figure 2)**

<table>
<thead>
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</tr>
<tr>
<td>Natural compound</td>
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</tr>
<tr>
<td>DNA topoisomerase inhibitor</td>
<td>7</td>
</tr>
<tr>
<td>RAR agonist</td>
<td>6</td>
</tr>
<tr>
<td>mTOR inhibitor</td>
<td>5</td>
</tr>
<tr>
<td>Typical anticancer</td>
<td>5</td>
</tr>
<tr>
<td>Reagent</td>
<td>5</td>
</tr>
<tr>
<td>ALKS inhibitor</td>
<td>4</td>
</tr>
<tr>
<td>Src Inhibitor</td>
<td>3</td>
</tr>
<tr>
<td>Plk inhibitor</td>
<td>3</td>
</tr>
<tr>
<td>CDK inhibitor</td>
<td>2</td>
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<tr>
<td>FGFR inhibitor</td>
<td>2</td>
</tr>
<tr>
<td>Multi-kinase inhibitor</td>
<td>2</td>
</tr>
<tr>
<td>ALK inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>HER2 inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>PDE inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>RAR agonist</td>
<td>1</td>
</tr>
<tr>
<td>Anti-infective agent</td>
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<tr>
<td>Total</td>
<td>76</td>
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were effective for FOP. Among these, we focused on mTOR signaling, because 5 mTOR inhibitors were identified, suggesting the feasibility of mTOR inhibition in this assay system. In particular, rapamycin and its analogs showed potent inhibition in the 5×A-Luc assay, even at 10 nM (Supplemental Figure 2). Furthermore, rapamycin and its analogs showed potent inhibition in the 5×A-Luc assay, demonstrating the essential role of mTOR signaling in this model. The transplantation of FOP-iMSCs into the gastrocnemius muscle of NOD/scid mice with C3H10T1/2 cells harboring Dox-inducible Activin-A showed HO when the mice were administrated Dox (Figure 5, A and B, and Table 5). As with the FOP-ACVR1 conditional transgenic mice, we observed safranin O-, von Kossa-, and COL1-positive cells, suggesting extraskeletal bone formation through an endochondral process (Figure 5C). Interestingly, i.p. or oral administration of rapamycin suppressed the HO triggered by Activin-A (Figure 5, Table 5, Supplemental Figure 7, C and D, and Supplemental Table 4). Since we observed a large number of anti-human–specific vimentin-positive cells in the rapamycin-treated group (Figure 5C), it seemed that the role of rapamycin in this model was not to kill the human cells but rather to suppress HO itself. Moreover, HO was suppressed, even when rapamycin treatment was started 2 weeks after transplantation and Dox administration (Supplemental Figure 7, C and D, and Supplemental Table 5); therefore, a window of opportunity for HO suppression would occur not before Activin-A is secreted, but rather after its secretion, when the Activin-A signal has been initiated to suppress HO itself. Taken together, rapamycin suppressed FOP-iPSC–derived extraskeletal bone formation in vivo, and mTOR inhibition was effective at suppressing HO in both FOP mouse models.

Enhanced mTOR signaling in chondrogenic induction of FOP-iMSCs. Although mTOR inhibition was effective at suppressing HO in vivo, the detailed mechanism was unclear. To clarify the critical role of mTOR signaling and its key components in the chondrogenic induction of FOP-iMSCs, we performed a loss-of-function study using siRNA. mTOR knockdown decreased GAG/DNA (Figure 6A) and Alcian blue staining (Figure 6B) in 2DCI assays of FOP-iMSCs, demonstrating the essential role of mTOR signaling. It is known that mTOR, a serine/threonine protein kinase, forms the catalytic subunit of 2 different protein com-

![Table 3. IC₅₀ values for everolimus, rapamycin, and temsirolimus in 5×A-Luc assay, 2DCI assay, and 3DCI assay (related to Figure 3)](https://doi.org/10.1172/JCI93521)

<table>
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<tr>
<th>IC₅₀</th>
<th>Everolimus</th>
<th>Rapamycin</th>
<th>Temsirolimus</th>
</tr>
</thead>
<tbody>
<tr>
<td>5×A-Luc</td>
<td>6.2 nM</td>
<td>0.4 nM</td>
<td>0.5 nM</td>
</tr>
<tr>
<td>2DCI</td>
<td>1.2 nM</td>
<td>0.2 nM</td>
<td>0.7 nM</td>
</tr>
<tr>
<td>3DCI</td>
<td>3.1 nM</td>
<td>0.5 nM</td>
<td>1.1 nM</td>
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Table 4. Number of FOP-ACVR1 conditional transgenic mice harboring HO (>20 mm² bone volume) triggered by Activin-A (related to Figure 4)

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of HO (&gt;20 mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>9/11</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>0/11</td>
</tr>
</tbody>
</table>
plexes, mTOR complex 1 (mTORC1, a rapamycin-sensitive complex) and mTOR complex 2 (mTORC2, a rapamycin insensitive complex), to orchestrate diverse functions (55–59). Therefore, we analyzed the importance of these complexes in chondrogenesis. As expected, we found that knockdown of RPTOR, a major component of mTORC1, decreased GAG/DNA and Alcian blue staining, but not RICTOR, a major component of mTORC2 (Figure 6, A and B). These data indicated a critical role of mTORC1, but not mTORC2, signaling in the 2DCI assays on FOP-iMSCs stimulated with Activin-A. We previously showed that in FOP-iMSCs, Activin-A transduces BMP signaling in addition to TGF-β signaling and that inhibition of either signal suppresses enhanced chondrogenesis in FOP-iMSCs (38) (Figure 1, B–E). Therefore, to determine whether rapamycin directly inhibits either BMP or TGF-β signaling, we performed Western blotting to analyze the phosphorylation of SMAD1/5/8 (cytoplasmic BMP signaling transducers) and SMAD2/3 (cytoplasmic TGF-β signaling transducers). Rapamycin did not directly inhibit BMP (Figure 6C) or TGF-β signaling (Figure 6D). Moreover, we found an upregulation of mTORC1 activity triggered by Activin-A during chondrogenesis in FOP-iMSCs compared with that observed in mutation-rescued FOP-iMSCs, in which the mutant ACVR1 was converted to WT (45). Phosphorylation of S6 (p-S6), a surrogate marker of mTORC1 activity (55–59), was enhanced in FOP-iMSCs

Table 5. Number of mice harboring HO (>20 mm³ bone volume) derived from FOP-iPSCs in vivo (related to Figure 5)

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of HOs (&gt;20 mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5/5</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Figure 1. Construction and characterization of the FOP-5×A-Luc assay system. (A) Construction of FOP-5×A-Luc-iPSCs. Luciferase, following the COL2A1 promoter and 5-repeats Aggrecan enhancer (5×A), was inserted into the pTrans1-3 vector to produce stably expressing cell lines by using Tol2 transposase from Japanese medaka fish. After cotransfection with pCAGGS-mT2TP (Tol2 transposase expression vector), the neomycin-resistant clone was selected and designated as FOP-5×A-Luc-iPSC. (B–E) Characterization of FOP-5×A-Luc-iMSCs. Luciferase activity (B), Aggrecan (ACAN) expression levels (qPCR analysis) (C), GAG/DNA ratios (D), and Alcian blue staining (E) of 2DCI assays. The cells were harvested 3 days (B), 7 days (C), or 6 days (D and E) after chondrogenesis induction was performed, with or without Activin-A and inhibitors (10 μM). Data represent the mean ± SEM. n = 4 (B–D). ***P < 0.001, by Dunnett’s multiple comparisons t test compared with the DMSO treatment control with Activin-A (B–D). Representative data of n = 3. Scale bar: 200 μm (E). SB, SB-43152.
In addition to rapamycin, we found that LY294002 and ipatasertib (AKT inhibitor), and BIRB 796 (p38MAPK inhibitor).

We evaluated several inhibitors, including LY294002 (PI3K inhibitor), which helps us to understand the signaling cascade of activated mTORC1 signaling. Through the 2DCI assay (Figure 6E and Supplemental Figure 8), we gained insight into the molecular mechanisms underlying the enhanced chondrogenesis observed in FOP-iMSCs. For this experiment, we performed an unbiased transcriptome analysis using a DNA microarray. Interestingly, we found that mTOR pathway–related genes were significantly changed in FOP-iMSCs and that mTORC1 activity is regulated by PI3K/AKT signaling.

ENPP2-mediated enhanced mTOR signaling in chondrogenic induction of FOP-iMSCs. Next, to gain insight into the molecular mechanisms underlying the enhanced chondrogenesis observed in FOP-iMSCs, we performed an unbiased transcriptome analysis using a DNA microarray. Interestingly, we found that mTOR pathway–related genes were significantly changed in FOP-iMSCs as compared with resFOP-iMSCs stimulated by Activin-A during the 2DCI assay (Figure 6E and Supplemental Figure 8). Finally, to evaluate the signaling cascade of activated mTORC1 signaling, we assessed several inhibitors, including LY294002 (PI3K inhibitor), ipatasertib (AKT inhibitor), and BIRB 796 (p38MAPK inhibitor). In addition to rapamycin, we found that LY294002 and ipatasertib, but not BIRB 796, inhibited the Activin-A–induced phosphorylation of S6 (Figure 6F). These data indicated an involvement of the PI3K/AKT/mTORC1 cascade. Taken together, our findings show that Activin-A activates mTORC1 signaling during chondrogenesis to a greater extent in FOP-iMSCs than in resFOP-iMSCs and that mTORC1 activity is regulated by PI3K/AKT signaling.

ENPP2-mediated enhanced mTOR signaling in chondrogenic induction of FOP-iMSCs. Next, to gain insight into the molecular mechanisms underlying the enhanced chondrogenesis observed in FOP-iMSCs, we performed an unbiased transcriptome analysis using a DNA microarray. Interestingly, we found that mTOR pathway–related genes were significantly changed in FOP-iMSCs as compared with resFOP-iMSCs 24 hours after chondrogenesis induction with Activin-A, but not with BMP-7 or TGF-β3 (Supplemental Figure 9). Therefore, Activin-A regulated mTOR signaling in the chondrogenesis of FOP-iMSCs by acting on global gene expression levels. Next, we explored the upstream regulators of mTORC1. Among the genes upregulated in FOP-iMSCs 24 hours after chondrogenesis induction with Activin-A but not with BMP-7 or TGF-β3 (Figure 7A), we found that ENPP2 was the most differentially expressed gene (Figure 7B). ENPP2 expression was increased after Activin-A–stimulated chondrogenesis induction, particularly in FOP-iMSCs (Figure 7C). ENPP2 is a secreted lysophospholipase that generates the lipid mediator lysophosphatidic acid (LPA), plays a key role in diverse physiological and pathological processes, and is known to activate mTOR signaling through LPA receptors (60, 61). The administration of ENPP2 inhibitors (HA130 and PF-8380) (Figure 7D) or knockdown of ENPP2 by siRNAs (Figure 7E) decreased the level of p-S6 induced by Activin-A. ENPP2 inhibitors also suppressed GAG/DNA in 2DCI assays of FOP-iMSCs stimulated by Activin-A (Supplemental Figure 10). Conversely, LPA treatment increased p-S6 levels in FOP-iMSCs (Figure 7F). Taken together, these results indicate that ENPP2 is an upstream regulator of the enhanced mTORC1 signaling triggered, at least partially, by Activin-A in FOP-iMSCs although further study is required for a more detailed understanding of the underlying mechanism.

Discussion

In this report, we clarified that mTOR signaling was enhanced in the chondrogenesis of FOP-iMSCs as compared with resFOP-iMSCs and that this aberrant signal was regulated by the Activin-A/FOP-ACVR1/ENPP2 cascade. It is known that mTOR signaling coordinates a variety of environmental inputs including energy, nutrients, stress, and growth factors. Thus, the dysregulation of mTOR signaling, i.e., the abrogation of homeostasis, causes many disorders including cancer, diabetes, neurodegeneration, and others (47, 48, 55–59). Rodgers et al. proposed that mTORC1 controls tissue regeneration by activating quiescent stem cells into an “alert state” to respond rapidly under conditions of injury and stress (62, 63). In FOP pathology, the mutation of ACVR1 appears to deviate this fine-tuned tissue homeostasis mechanism. Activin-A is strongly expressed in wounded regions and promotes the regeneration of damaged tissue (64–66). In FOP, however, we found what we believe to be a new signaling pathway for Activin-A that initiates HO formation by mistransducing BMP signaling. MSCs, which are cells of origin of HO in FOP (67, 68), respond abnormally to an Activin-A–evoked signal by taking on an osteo/chondrogenic lineage that is consistent with the response to bone fracture. Because Activin-A activates mTOR signaling in FOP, this response by MSCs could be related to the alert state. Rodgers et al. also showed that HGF signaling is necessary for transition to the alert state as a putative upstream regulator of mTORC1 signaling. On the other hand, we show that ENPP2-mediated mTORC1 activation is the key event in aberrant chondrogenesis. These observations suggest that mTOR signaling is precisely regulated by the cell type and stimulant. To fully understand mTORC1 signaling in the enhanced chondrogenesis of FOP, it is necessary to investigate the downstream effectors of mTORC1. Furthermore, as we previously discussed (38), because Activin-A does not bind to GREM, an inhibitor of BMPs (14, 16, 69) that is upregulated in FOP-iMSCs stimulated with Activin-A, BMP signaling does not properly negatively regulate Activin-A. Given these results, we propose that the mechanistic cause of FOP pathology involves the activation of mTOR signaling by Activin-A and the failed negative regulation by BMP signaling.

Although much research has been devoted to mTOR signaling, there are a limited number of reports about the relationship between mTOR signaling and chondrogenesis. During chicken embryonic development, the mechanical activation of mTOR is required for chondrocyte proliferation and chondrogenesis during...
showed that mTOR inhibition potently suppressed the enhance-
chondrogenesis and are mostly consistent with our study, which
ulates chondrogenesis, albeit by distinctive mechanisms. These
and mouse studies suggest that mTOR signaling positively mod-
matrix, without changes in chondrocyte proliferation or survival,
Furthermore, a reduction in cell size and the amount of cartilage
mTORC1 signaling diminishes embryonic skeletal growth, causing
activated during limb cartilage development, and the disruption of
TSC1
mTORC1 via
On the other hand, another report showed that hyperactivation of
and the expression of a housekeeping gene (β-actin), respectively.
On the other hand, another report showed that hyperactivation of
mTORC1 via TSC1 gene deletion in chondrocytes blocked chondro-
bone development (70). In mouse embryos, mTORC1 signaling is
activated during limb cartilage development, and the disruption of
mTORC1 signaling diminishes embryonic skeletal growth, causing
severe delays in chondrocyte hypertrophy and bone formation (71).
Furthermore, a reduction in cell size and the amount of cartilage
matrix, without changes in chondrocyte proliferation or survival,
cause a reduction in the growth of cartilage (71). Both the chicken
and mouse studies suggest that mTOR signaling positively mod-
ulates chondrogenesis, albeit by distinctive mechanisms. These
observations support the notion that mTOR signaling promotes
chondrogenesis and are mostly consistent with our study, which
showed that mTOR inhibition potently suppressed the enhance-
ment of chondrogenesis in FOP-iMSCs or HO in vivo (Figures
3–5 and Supplemental Figures 4–7). In our 2D and 3DCI assays
of Activin-A-stimulated FOP-iMSCs, mTOR inhibitors clearly
decreased both GAG/DNA and the expression levels of chondro-
genesis markers (Figure 3 and Supplemental Figure 4), indicating
of Activin-A–stimulated FOP-iMSCs, mTOR inhibitors clearly
rate its future clinical trial for FOP. Interestingly, noting the crit-
role of the mTOR pathway in vivo, although species differences
should be considered, since FOP-Acvr1 heterozygous mice showed
perinatal lethality (50). Another important aspect of our study is
use of a bioactive library that contains currently marketed drugs.
The fact that rapamycin is an approved drug (46–48) should accel-
erate its future clinical trial for FOP. Interestingly, noting the crit-
ical roles of Hif1α on normal chondrogenesis, Agarwal et al. also
found that rapamycin decreased extraskeletal bone formation
by inhibiting HIF1α in 3 HO mouse models (31). Also of note, the
expression levels of HIF1α and several related downstream genes
were not upregulated in FOP-iMSCs compared with resFOP-
iMSCs (Supplemental Figure 11), suggesting that the HIF1α pathway
is not related to the Activin-A/FOP-ACVR1/mTOR signaling path-
way in FOP-iMSCs, although hypoxia occurs during HO, as dis-
FOP-iPSC–based in vivo FOP model is unique, in that it enabled
us to test the effect of drug candidates on human patient–derived
cells in an in vivo environment. However, even though iPSC–based
in vivo models have several advantages, there are limitations as
well. For example, they require transplantation of the cells into
immunodeficient mice, but many types of immune cells contribute
to the HO in FOP (73). To ensure the importance of mTOR signal-
ing in FOP pathology, we also established FOP-ACVR1 conditional
transgenic mice. This FOP mouse model confirmed the critical
role of the mTOR pathway in vivo, although species differences
should be considered, since FOP-Acvr1 heterozygous mice showed
perinatal lethality (50). Another important aspect of our study is
use of a bioactive library that contains currently marketed drugs.

Figure 3. mTOR inhibitors suppress the chondrogenic induction of FOP-iMSCs triggered by Activin-A. (A–
C) Concentration-dependent assays of everolimus, rapamycin, and temsirolimus. 5×A-Luc assay (A), 2DCI
assay (B), and 3DCI assay (C) in FOP-iMSCs triggered by Activin-A. Cells were harvested 4 days (A), 7 days
(B), or 21 days (C) after chondrogenesis induction was induced with Activin-A, with or without inhibitors.
(D and E) Alcian blue staining of FOP-iMSCs in 2DCI (D) and 3DCI (E) assays stimulated by Activin-A. Each
compound was used at 100 nM. Scale bars: 200 μm (D) and 100 μm (E). Data represent the mean ± SEM. n =
4 (A) and n = 3 (B and C). Results are representative of at least 2 independent experiments.
discussed in several studies (31, 32). Another important role of mTOR signaling is that of mediating inflammatory responses (74, 75), which might have an impact during the early stages of FOP in vivo (73). However, our in vitro chondrogenesis induction experiments using iMSCs without immune cells clearly showed that mTOR signaling is activated downstream of FOP-ACVR1 signaling (Figure 6 and Supplemental Figure 8) and that mTOR inhibitors potently suppressed the enhanced chondrogenesis of FOP-iMSCs (Figure 3).

**Figure 4. Rapamycin suppresses Activin-A–triggered HO in FOP-ACVR1 conditional transgenic mice.** Schematic of FOP-ACVR1 (R206H) conditional transgenic mice. (B–D) Activin-A injection and oral administration of Dox-induced HO, which was suppressed by i.p. administration of 5 mg/kg rapamycin (once daily, 5 times a week) in FOP-ACVR1 transgenic mice. The mice were analyzed 3 weeks after Activin-A injection and rapamycin administration. (B) X-ray and μCT findings. (C) Average heterotopic bone volume. (D) Histological analysis of the Activin-A–injected region. H&E, safranin O (acidic proteoglycan), von Kossa (calcium), and anti-COL1 (bone marker) staining. Scale bars: 10 mm (B) and 100 μm (D). Data represent the mean ± SEM. n = 11 (vehicle or rapamycin). ***P < 0.001, by Student’s t test compared with the vehicle-treated group.
Methods

Cell culture. iPSCs were maintained in primate embryonic stem (ES) cell medium (ReproCELL) supplemented with 4 ng/ml recombinant human FGF2 (Wako Pure Chemical). To activate the production of induced neural crest cells (iNCCs), mTeSR1 medium (STEMCELL Technologies) was used for the feeder-free culturing of iPSCs. The induction and maintenance of iNCCs and iMSCs derived from iPSCs were previously described (43, 45) (Supplemental Figure 1A). Briefly, iNCCs were induced in chemically defined medium (CDM) supplemented with 10 μM SB-431542 and 1 μM CHIR99021 for 7 days. iNCCs

and Supplemental Figure 4). We therefore propose that targeting mTOR signaling can modulate aberrant FOP-ACVR1 signaling, hypoxic signaling, and inflammatory signaling during HO. Therefore, mTOR inhibitors have the potential to potently suppress HO in FOP patients by inhibiting these 3 pathways. Given these serendipitous findings, we believe that rapamycin is a promising drug candidate for the treatment of FOP and that an iPSC-based drug discovery platform, including iPSC-based HTS and in vivo models, offers a remarkable advantage for exploring candidate drugs with a higher probability of reaching clinical trials.

Figure 5. Rapamycin suppresses HO derived from FOP-iPSCs in vivo. Administration of 5 mg/kg rapamycin (i.p., once daily, 5 times a week) suppressed Activin-A–triggered HO derived from FOP-iMSCs. The mice were analyzed 6 weeks after transplantation and rapamycin administration. (A) X-ray and \( \mu \)CT findings. (B) Average heterotopic bone volume. (C) Histological analysis of the cell-transplanted region. H&E, safranin O, von Kossa, anti-COL1, and anti-human vimentin staining. Scale bars: 10 mm (A) and 100 μm (C). Results represent the mean ± SEM. n = 5 (vehicle) or 4 (rapamycin). **P < 0.01, by Student’s t test compared with the vehicle-treated group. Data are representative of 3 independent experiments.
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were maintained in CDM supplemented with 10 μM SB-431542, 20 ng/ml FGF2, and 20 ng/ml recombinant human EGF (R&D Systems), and we used up to 20 passages in this study. iMSCs were induced and maintained in mMEM (Invitrogen, Thermo Fisher Scientific) supplemented with 10% (v/v) FBS (Nichirei), 5 ng/ml FGF2, and 0.5% penicillin and streptomycin (Invitrogen, Thermo Fisher Scientific). The FOP-iMSCs used in this study (previously described as vFOP4-1 [ref. 44]) harbor the R206H heterozygous mutation in ACVR1, and gene-corrected resFOP-iMSCs were generated by BAC-based homologous recombination. These cells fulfilled several criteria for iPSCs including the expression of pluripotent markers, teratoma formation, normal karyotype, and morphology. Growth and gene expression profiles of the resFOP-iPSC clones were indistinguishable from the original FOP-iPSCs (44). However, remarkably distinct response profiles to Activin-A were observed (38). Murine multipotent mesenchymal C3H10T1/2 cells expressing Dox-inducible human INHBA (C3H-DoxOn-hINHBA) were maintained in DMEM (Nacalai Tesque) supplemented with 10% FBS and 1 mM Na-pyruvate (Thermo Fisher Scientific) and used for an Activin-A–induced HO model transplanted with FOP-iMSCs as previously reported (38).

Figure 6. Enhanced mTOR signaling in chondrogenic induction of FOP-iMSCs. (A and B) Knockdown experiments of mTOR complex components assessed by GAG/DNA (A) and Alcian blue staining (B) in a 20CI assay of FOP-iMSCs. Scale bar, 200 μm. (C and D) Phosphorylation of SMAD1/5/8 (C) and SMAD2/3 (D) in FOP-iMSCs. Serum-starved FOP-iMSCs were pretreated with 10 nM rapamycin (Rapa), 1 μM DMH1, or 1 μM SB-431542 for 1 hour, and after a 1-hour stimulation with Activin-A, the cells were harvested. (E) p-S6 was enhanced in FOP-iMSCs compared with resFOP-iMSCs in 20CI assays, p-S6 was enhanced in Activin-A–stimulated FOP-iMSCs compared with levels detected in resFOP-iMSCs. The strength of resFOP at each time point was set at 1. (F) Enhanced p-S6 was inhibited by mTOR (Rapa), PI3K (LY), or AKT (Ipa) inhibitors 24 hours after treatment with Activin-A and inhibitors in FOP-iMSCs. Treatment with 10 μM DMH1, 10 μM BIRB 796 (BIRB), 10 μM LY294002 (LY), 10 μM ipatasertib (Ipa), or 10 nM rapamycin was used. Results represent the mean ± SEM. n = 3 (A and B, C–F). *P < 0.05, **P < 0.01, and ***P < 0.001, by Dunnett’s multiple comparisons t test compared with the DMSO-treated control stimulated with Activin-A (C, D, and F); with the siRNA-transfected negative control, with or without Activin-A stimulation (A); and by Student’s t test compared with resFOP-iMSCs stimulated with Activin-A (E). Data are representative of 2 independent experiments (A, B, E, and F).

Reagents. Activin-A, BMP-7, and TGF-β3 were purchased from R&D Systems. Rapamycin and everolimus were purchased from MedChem Express. SB-431542 and temsirolimus were purchased from Sigma-Aldrich. LPA and LY294002 were purchased from Cayman Chemical. Ipatasertib and BIRB 796 were purchased from Selleck Chemicals. DMH1, HA130, and PF-8380 were purchased from Tocris Bioscience, EMD Millipore, and Ark Pharm, respectively. Activin-A, BMP-7, and TGF-β3 were dissolved according to the manufacturer’s protocols and used at 100 ng/ml (Activin-A and BMP-7) or 10 ng/ml (TGF-β3), unless otherwise noted.

Chemical libraries. All chemical libraries were purchased from the suppliers listed in Table 1. All compounds were bioactive and/or annotated, with the exception of compounds from Analyticon Discovery, which mainly includes natural compounds.

Generation of FOP-5×A-Luc-iPSCs. Luciferase (luc+; Promega) following the COL2A1 promoter and 5-repeats Aggrecan enhancer (49) was inserted into pTrans1-3 (76), which enabled us to easily produce stable expressing cell lines using Tol2 transposase from Japanese medaka fish (Oryzias latipes) (77,78) (pTrans1-3/5×A-Luc, Figure 1A). pTrans1-3/5×A-Luc and pCAGGS-mT2TP (Tol2 transposase expression vector) were cotransfected into FOP-iPSCs by FuGENE HD (Promega) according to the manufacturer’s protocol, and the neomycin-resistant clone (100 μg/ml) was selected and used as FOP-5×A-Luc-iPSCs. pTrans1-3 included SAR-CH4, an insulator module from...
Figure 7. ENPP2 mediates enhanced mTOR signaling in chondrogenic induction of FOP-iMSCs. (A) Venn diagram of genes highly upregulated in FOP-iMSCs compared with resFOP-iMSCs 24 hours after chondrogenesis induction stimulated with Activin-A, BMP-7, or TGF-β3. (B) Genes highly upregulated in FOP-iMSCs 24 hours after chondrogenesis induction stimulated with Activin-A, but not with BMP-7 or TGF-β3. (C) Expression of ENPP2 during the chondrogenic induction of FOP-iMSCs and resFOP-iMSCs. The expression level of resFOP (D (0 h) was set at 1. (D and E) ENPP2 inhibitors (HA, HA130; PF, PF-8380) (D) and ENPP2 knockdown (E) reduced p-S6 levels. (D) The cells were harvested 24 hours after treatment with Activin-A and ENPP2 inhibitors, and (E) siRNA-transfected cells were harvested 48 hours after treatment with Activin-A. Rapa, 100 nM rapamycin; HA, 10 μM HA130; PF, 10 μM PF-8380. (F) Serum-starved FOP-iMSCs were treated with 100 μM LPA, and 30 minutes after stimulation, the cells were harvested. Results represent the mean ± SEM. n = 1 (A and B) and n = 3 (C–F). *p < 0.05, **p < 0.01, and ***p < 0.001, by Student’s t test compared with LPA (-) control (F), by Dunnett’s multiple comparisons t test compared with FOP-iMSCs stimulated with Activin-A (D), or with negative control siRNA-transfected FOP-iMSCs stimulated with Activin-A (E). Data are representative of 2 independent experiments (D and F).
heterozygous tetO-FOP-ACVR1 allele were used to induce the FOP-ACVR1 gene. Genomic DNA was extracted from the tail of each mouse using a DNeasy Blood and Tissue Kit (QIAGEN) and subjected to genotype analysis. PCR was performed using KOD-Plus Neo (TOYOBO) according to the manufacturer’s instructions. The following PCR primers were used: for Col1a1-FOP-ACVR1: oIMR6724_Col1-FOP, CCTCCATGTGTGACCAAGG; oIMR6725_Col1-FOP, GCACAGCATTGCGGACATGC; and oIMR6726_Col1-FOP, GCGAGCCTGTGCCTCCATGTGTGACCAAGG. DNA was extracted from the tail of each mouse and subjected to TRI/3D-BON software (Ratoc System Engineering) for analysis 11–16 days after injection.

Cardiotxin-induced HO model in human FOP-ACVR1 conditional transgenic mice. Male mice (F3–F5) offspring of chimeric mice, age- and BW-matched between groups, were used between 16 and 21 weeks of age. Mice were administered 2 mg/ml Dox in their drinking water supplemented with 10 mg/ml sucrose to induce FOP-ACVR1. Cardiotxin (9.1 μg/mouse; latoxan) was injected into the right gastrocnemius muscle to initiate skeletal muscle injury and subsequent heterotopic bone formation (50). Rapamycin (16% DMSO in 0.5 w/v% methylcellulose 400 cP) was administered i.p. once a day, 5 times a week. Mice were analyzed 4 weeks after injection. For x-ray images, mice were anesthetized with isoflurane (5% for induction, 2%–3% for maintenance; Abbvie), immobilized, and x-rayed using μFX-1000 (Fujifilm) or DX-50 (Faxitron Bioptics). μCT images were scanned using x-ray CT systems (inspeXio SMX-100CT; Shimadzu) and analyzed by TRI/3D-BON software (Ratoc System Engineering) according to the manufacturer’s instructions. Four weeks after injection, the injected sites were harvested, fixed with 4% paraformaldehyde for twenty-four hours, embedded in paraffin, and sectioned and stained with H&E, von Kossa, safranin O, human-specific anti-vimentin antibody, or anti-collagen I antibody as previously described (38, 80).

Activin-A-induced HO model in human FOP-ACVR1 conditional transgenic mice. Male mice (F3–F5) offspring of chimeric mice, age- and BW-matched between groups, were used between 13 and 14 weeks of age. Activin-A (13.3 μg/mouse) was injected into the right gastrocnemius muscle to initiate skeletal muscle injury and subsequent heterotopic bone formation (50). Rapamycin (16% DMSO in 0.5 w/v% methylcellulose 400 cP) was administered i.p. once a day, 5 times a week. Mice were analyzed 3 weeks after injection.

BMP-7-induced HO model mice. BMP-7 (2 μg/mouse) was injected into the right gastrocnemius muscle of 6- to 8-week-old male C57BL/6Jcd (CLEA Japan), and compounds were administered once a day, 5 times a week (orally), or every day (i.p.). Mice were analyzed 11–16 days after injection.

Activin-A-induced HO model transplanted with FOP-IMSCs. FOP (right leg) and resFOP-IMSCs (left leg) (4 × 10^5 cells, respectively) were transplanted into the gastrocnemius muscle of 6- to 8-week-old NOD/ShiJic-scid Jcl (NOD/SCID) mice (CLEA Japan) with C3H-Dox-On-hINHBA (5 × 10^5 cells), which can continuously expose Activin-A to the transplanted IMSCs in vivo (38). In the Dox-induced group, 1 mg/ml Dox (Sigma-Aldrich) was administered via the drinking water with 10 mg/ml sucrose (Nacalai Tesque) for 2 weeks after transplantation. Compounds were administered i.p. or orally once a day, 5 times a week. Six to eight weeks after transplantation, the transplanted cells were harvested, fixed with 4% paraformaldehyde for twenty-four hours, embedded in paraffin, and sectioned and stained with H&E, von Kossa, safranin O, human-specific anti-vimentin antibody, or collagen I antibody as previously described (38, 80).

Quantitative PCR analysis. Total RNA was purified with an RNase-Free Kit (QIAGEN) and treated with a DNase-One Kit (QIAGEN) to remove genomic DNA. Total RNA (0.3 μg) was reverse transcribed for single-stranded cDNA using random primers and Superscript III Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed with Thunderbird SYBR qPCR Mix (TOYOBO) and analyzed with the StepOne Real-Time PCR System (Applied Biosystems). The primer sequences are described in Supplemental Table 7. 3D chondrogenic differentiated pellets were homogenized using a Multi-Beads Shocker (Yasui Kikai) according to the manufacturer’s instructions. All data (relative expression) were corrected by β-actin.

Microarray experiments. 2D chondrogenesis induction was performed in FOP- and resFOP-IMSCs stimulated with 100 ng/ml Activin-A, 100 ng/ml BMP-7, or 10 ng/ml TGF-β3, and after incubation for 6, 24, or 48 hours or 7 days, mRNA was extracted. RNA was reverse transcribed, biotin labeled, and hybridized to the GeneChip Human Gene 1.0 ST Expression Array (Affymetrix), which was subsequently washed and scanned according to the manufacturer’s instructions. Raw CEL files were imported into GeneSpring GX 12.6.1 software (Agilent Technologies), and the expression values were calculated with the RMA16 algorithm. Pathway analysis was performed using Ingenuity Pathway Analysis (QIAGEN). Array data were deposited in the NCBI’s Gene Expression Omnibus (GEO) database (GEO GSE90638).

Western blotting. SDS-PAGE and blotting with whole-cell lysates were performed by standard procedures. Protein bands were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized using the Bio-Rad Molecular Imager Chemi-Doc XRS+ with Image Lab software (Bio-Rad). The antibodies used in this study are described in Supplemental Table 8. All data (relative intensity) were corrected by β-actin or total SMADs.

GAG value. GAG content was quantified in pellets with the Blyscan Glycosaminoglycan Assay Kit (Bicolor). DNA content was quantified using the PicoGreen dsDNA Quantitation Kit (Thermo Fisher Scientific).

IHC. Paraffin-embedded sections were deparaffinized, and for human-specific anti-vimentin antibody, antigen retrieval was performed by autoclaving (105°C, 10 min). Samples were blocked with Blocking One (Nacalai Tesque) for 60 minutes and then incubated with human-specific anti-vimentin antibody (Abcam) or anti-collagen I antibody (Novus Biologicals) diluted in Can Get Signal Immunostain Solution B (Toyobo) for 16 to 18 hours at 4°C. Next, samples were washed several times in 0.2% Tween-20 (Sigma-Aldrich) in PBS and incubated with goat anti-rabbit IgG (H+L) secondary antibody or Alexa Fluor 488 or Alexa Fluor 555 conjugate (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. Samples were observed by BZ-9000E (KEYENCE). Samples were washed and scanned according to the manufacturer’s instructions.

Statistics. The statistical significance of all experiments was calculated using 2-tailed Student’s t test or 1-way ANOVA for Dunnett’s multiple comparisons test. All statistical tests were performed using GraphPad Prism 6 (GraphPad Software). P values of less than 0.05 were considered statistically significant.

Study approval. 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 consent was obtained from the human subjects.
informed consent was provided by each donor. All animal experiments were approved by the institutional animal committee of Kyoto University.

**Author contributions**

K. Hino, JT, and MI conceived and designed the experiments. K. Hino, K. Horigome, MN, SK, SN, CZ, Vj, and AO performed the experiments. K. Hino, K. Horigome, MN, and AO analyzed the data. K. Horigome, YY, and KK contributed the reagents, materials, and analysis tools. K. Hino, JT, and MI wrote the manuscript. All authors read and approved the final manuscript.

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