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Review

Insights into the biology of fibrodysplasia ossificans progressiva using patient-derived induced pluripotent stem cells☆

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ABSTRACT

The demand for development of new drugs remains on the upward trend because of the large number of patients suffering from intractable diseases for which effective treatment has not been established yet. Recently, several researchers have attempted to apply induced pluripotent stem cell (iPSC) technology as a powerful tool for studying the mechanisms underlying the onset of various diseases and for new drug screening. This technology has made an enormous breakthrough, since it permits us to recapitulate the disease phenotype *in vitro*, outside of the patient's body. Here, we discuss the latest findings that uncovered a mechanism underlying the pathology of a rare genetic musculoskeletal disease, fibrodysplasia ossificans progressiva (FOP), by modeling the phenotypes with FOP patient-derived iPSCs, and that discovered promising candidate drugs for FOP treatment. We also discussed future directions of FOP research.

Contents

1. Introduction

Discovering a new drug requires tremendous efforts. Typically, it involves a long period of over ten years and massive costs, such as over a hundred million US dollars [1–5]. After the long-term developmental process including identification of the target molecule, screening and optimization of the compound, pharmacokinetic test, preclinical test, and clinical trial, the stage of a new drug application is finally reached. Most of the compounds drop out before the preclinical phase; thus, the probability of a compound

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reaching market as a new therapeutic agent is extremely low [3,6–11]. Of late, significant advances in computational sciences, such as the appearance of super-computer, make it possible to introduce several innovative technologies in the field of medical science. One such example is virtual *in silico* screening that simulates and evaluates compound libraries computationally [12]. However, the number of new drug approvals is gradually declining each year [13–16].

Induced pluripotent stem cell (iPSC) technology can also be used for drug discovery research, besides regenerative medicine [17–20]. Taking advantage of its pluripotency, various types of cells constituting our body can be differentiated from iPSCs and used for drug safety, toxicity, and pharmacokinetic assays. Moreover, this technology can also be applicable to research on drug discovery in case of intractable hereditary diseases for which no effective treatment exists. For this purpose, disease-specific iPSCs with a genetic background of a disease can be generated from patient-derived cells [21,22]. This is a revolutionary advance as it enables us to model the disease phenotypes *in vitro* outside of the patient's body and to study the mechanisms underlying the onset of disease. Although most of these studies used to depend on animal models such as mice, the disease-specific iPSCs can also help to research on diseases that have significant species differences between humans and mice [23–26].

Fibrodysplasia ossificans progressiva, FOP, is a rare genetic disease characterized by endochondral heterotopic ossification in soft tissues, including skeletal muscles, ligament, and tendon, where a bone is not typically observed (Fig. 1) [27–32]. Approximately 90% of FOP patients share an R206H (617G > A) point mutation in the intracellular glycine- and serine-rich domain of ACVR1, a type I receptor for bone morphogenetic proteins (BMPs) [33–37], and the excessive transmission of the BMP signaling by mutant ACVR1 results in the bone formation ectopically [34,38–50]. This extra-skeletal ossification is often initiated between the infant and childhood stage; mean age at FOP diagnosis is 6.9 years [51], sometimes accompanied by the hallux valgus, baldness, and hearing impairment. Also, it is known that the ossification often progresses dramatically followed by flare-up, inflammatory subcutaneous soft tissue swelling, due to inflammation such as trauma, surgical invasion, and infection. Ossification starts mainly from the trunk region and gradually tends to spread towards the periphery, thereby progressively decreasing the patient's exercise ability and function. The bone formation in tissues related to respiration, such as thorax, or in tissues related to chewing could lead to lifespan-shortening [27–32]. At present, no effective treatment for FOP has been approved. In this review, we discuss the latest findings in FOP research using patient-derived iPSCs.

2. Studying FOP with patient-derived iPSCs

2.1. Modeling FOP phenotypes with patient-derived iPSCs

It is known that different donor tissues for generating iPSCs could influence the nature of iPSCs because of the epigenetic memory [52]. Thus, the robust differentiation method of human iPSC-derived target cells and the generation of genetically matched control iPSCs are needed to establish a successful *in vitro* model using patient-derived iPSCs for the disease phenotypes.

During our body plan formation, skeletal tissues such as cartilages and bones originate from multiple developmental origins, including neural crest cells, paraxial mesoderm cells, and lateral plate mesoderm cells. These embryonic sources mainly give rise to skeletal tissues in the cranial, trunk, and limb region, respectively, at the postnatal state [53–60]. Several researchers have been trying to generate iPSCs-derived cartilage and bone through various developmental origins step by step, or directly from iPSCs [61–65]. Our previous studies have reported the establishment of neural crest cell-derived and paraxial mesoderm cell-derived chondrocytes from human iPSCs [66–68].

Furthermore, we applied bacterial artificial chromosome (BAC)-based homologous recombination technique to correct the FOP mutation (617 G > A) existing in exon 7 of ACVR1, and reported the establishment of mutation-rescued iPSCs (resFOP-iPSCs) from FOP patient-derived iPSCs [69]. The resFOP-iPSCs could be used as control iPSCs because they have the same genetic background as FOP-iPSCs.

Using these materials, we elucidated the biology of FOP by modeling the phenotypes *in vitro*. FOP-iPSCs showed increased mineralization and cartilage formation compared to control healthy iPSCs [38]. These results indicate that the FOP ACVR1 mutation (R206H) favors chondrogenesis and increases mineral deposition *in vitro*. Moreover, the mineralization phenotypes could be suppressed with a small molecule inhibitor of BMP signaling, DMH1. We also demonstrated that enhanced *in vitro* chondrogenic ability of neural crest-derived mesenchymal stromal cells (MSCs; positive for CD44, CD73, and CD105), induced from FOP-iPSCs, was transcriptionally distinguishable from that of resFOP-iPSCs. SMAD1/5/8, SMAD2/3, and ERK1/2 pathways were significantly activated in

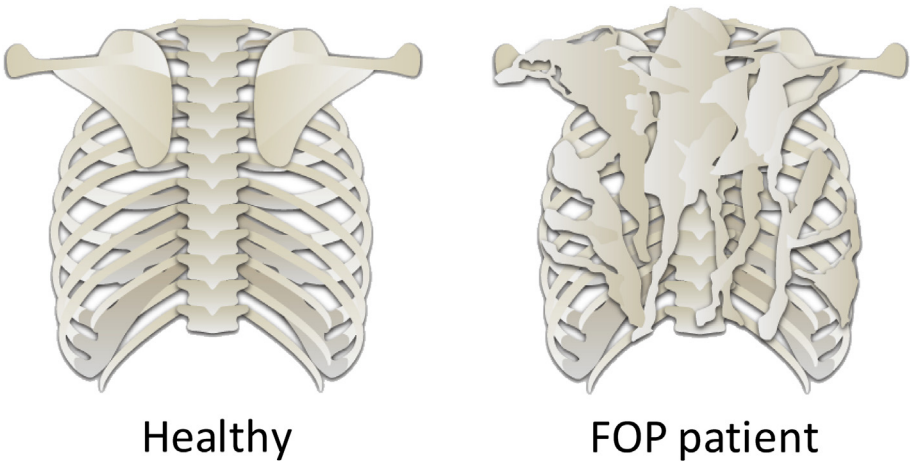

Fig. 1. Ectopic bone formation in FOP patients. Schematic view of ectopic bone formation observed in FOP patients. This figure is provided by Masaya Todani (Center for iPS Cell Research and Application) and edited by authors.

FOP-iPSC-derived MSCs (FOP-MSCs) [69]. We also performed a genome-wide transcriptional analysis, and identified PAI1 and MMP1 as key genes that are possibly associated with FOP onset and phenotypes, by demonstrating that these were activated in FOP-MSCs compared to resFOP-iPSC-derived MSCs (resFOP-MSCs), and played a critical role during chondrogenesis.

Normally, the ectopic ossification in FOP patients does not appear at birth but starts to develop during childhood. We established the iPSC differentiation method of sclerotome (SCL)-derived embryonic chondrocytes and MSCs-derived chondrocytes through the paraxial mesoderm, and compared the chondrogenic ability using FOP-iPSCs/resFOP-iPSCs [67]. Consequently, enhanced chondrogenesis was observed in the MSC-derived chondrogenic pathway as previously reported [69,70] but not in the SCL-derived embryonic chondrogenic pathway. These observations imply the cell-type specificity of FOP phenotypes, which possibly reflects the onset of FOP.

2.2. Mechanisms underlying the phenotype of FOP

It has been shown that mutation in ACVR1, encoding a type I receptor for BMP, is responsible for FOP [71]. There are several proposed theories on the mechanism by which excessive BMP signaling is transmitted downstream. In particular, there are two major theories, stating that the signal is over-transmitted by the BMP ligand binding to the mutant ACVR1, and that the signal is constitutively activated regardless of the binding with BMP. However, the embryonic and postnatal skeletogenesis of FOP patients is nearly normal although BMP signaling has a pivotal role during human body development [28–30,32].

As an alternative to these canonical theories, in 2015 we advocated a new hypothesis that a ligand which does not belong to the BMP family binds to mutant ACVR1 and then transmits BMP signaling instead. Due to this, bone/cartilage formation is abnormally enhanced, leading to ectopic ossification in FOP patients [70]. To screen ligands that activate BMP signaling through only mutant ACVR1 but not wild-type ACVR, we introduced luciferase reporter construct to both FOP-MSCs and resFOP-MSCs for detecting BMP activity. These cells were treated with 27 different ligands having a structure similar to BMP ligands, which belong to the TGF-β superfamily. Then, the luciferase activity was measured in these treated cells. Consequently, BMP signaling was activated to higher levels in FOP-MSCs by the addition of several BMPs such as BMP 6 and BMP 7, as reported so far, but the ratio was approximately 1.4 times compared with resFOP-MSCs. Surprisingly, it was found that the ratio dramatically increased more than 4 times only by activin A, which belongs to the TGF-β superfamily, similar to BMPs. When knocking down mutant ACVR1 in FOP-MSCs, activation of BMP signaling was not observed. When mutant ACVR1 was overexpressed in another bone lineage cell; U2OS cells, BMP signaling was activated in response to activin A. Furthermore, investigating the effect of activin A on differentiation of iPSC-derived chondrocytes revealed that the cartilage formation is enhanced by activin A administration in FOP-MSCs. In addition, an ectopic bone was formed after FOP-MSC transplantation with activin A-expressing cells into immunodeficient mice (Fig. 2).

Based on the above findings, it is elucidated that abnormal BMP signaling transduction through mutant ACVR1 is caused by activin A, a molecule that generally transmits TGF-β signaling and contributes to inflammatory responses. Similar results were also reported from another group [72]. This new finding supports and coincides with the fact that patients usually show FOP symptoms after trauma and/or inflammation. These discoveries also uncovered a part of the pathological mechanism of FOP, which causes ectopic bone tissue formation, and suggests a potential utilization of anti-activin A-related compound as a drug candidate for FOP.

2.3. Discovering the candidate drugs for FOP

Given the adverse prognosis and the difficulty in surgical therapy for FOP, developing an effective drug is strongly desired. Thus, several researchers have been struggling for a breakthrough since a long time. As candidate drugs for the disorders, which accompanied heterotopic ossification, several chemical compounds are proposed e.g. inhibitors of BMP type I receptors, such as LDN193189 and dorsomorphin which repress SMAD1/5/8 phosphorylation [73,74]; RARγ agonists, which prevent the expression of SMAD1/5/8 [75]; hypoxia-inducible factor-1α inhibitor, which inhibit the production of mesenchymal condensations [76]. Among them, Clementia Pharmaceuticals Inc. have started a clinical trial that investigate the curative effect of a RARγ agonist; palovarotene on FOP patients.

Also, we reported a novel chemical screening system adopting iPSC technologies and revealed a promising drug candidate, rapamycin (international nonproprietary name; sirolimus), which could prevent the development of ossification in FOP patients [77]. We established the screening system using FOP-MSCs harboring luciferase following 5-repeats aggrecan enhancers to monitor the activity of chondrogenesis, and performed an initial screening against our chemical library, containing 6809 compounds and assessed the inhibitory effect on cartilage differentiation of FOP-MSCs. Then, a second screening was done using 549 compounds that had been evaluated to possess a certain effect at the first screening, and consequently, 76 compounds were shortlisted after considering their cytotoxicity. These hit compounds include RARγ agonists and BMP signaling inhibitors that have been reported previously [73–75], but also include five mTOR inhibitors, suggesting the feasibility of mTOR inhibitors in our system. Several researchers have addressed the correlation of mTOR signaling and chondrogenesis as Chen and Long reported that mammalian target of rapamycin complex 1 (mTORC1) signaling controls skeletal growth through stimulation of protein synthesis in chondrocytes [78]. Also, Lim et al., reported that Bmp receptor type-1a controls osteoblast activity through mTORC1 signaling in mice, thus it is acceptable that mTOR signaling seems to be a downstream effector of Bmp signaling in skeletogenesis [79]. We subsequently investigated the effect of mTOR inhibitors using a model of FOP-MSC transplant into mice to form ectopic bone, and consequently found that the formation of ectopic bone by activin A stimulation was suppressed by administering rapamycin (Fig. 3). Therefore, these studies demonstrated the possible application of rapamycin to treat FOP patients. Based on these research achievements, Kyoto University Hospital started an investigator-initiated clinical trial that tests the curative effect on FOP patients in September 2017. The efficacy and safety are currently being assessed by multicenter randomized double-blind placebo-controlled comparison test followed by open-label continuous administration.

In addition, we proposed another drug candidate for FOP with a different model of chemical screening [80]. It is reported that approximately half of FOP patients experienced the progression of ectopic bone formation without apparent flares or injury [51]. We thus focused on the constitutive activity of mutated ACVR1 (FOP-ACVR1) as well, and developed a high-throughput screening system using a murine chondrogenic cell line, ATDC5, with doxycycline-inducible human FOP-ACVR1. As several reports demonstrated, ATDC5 is known to increase alkaline phosphatase (ALP) expression following BMP stimulation, and ALP activity can be monitored by a chromogenic phosphatase substrate in a chemical screening format. Consequently, two candidate compounds were identified from screening of 4892 compounds that suppressed the enhanced chondrogenesis in FOP-iPSCs and that

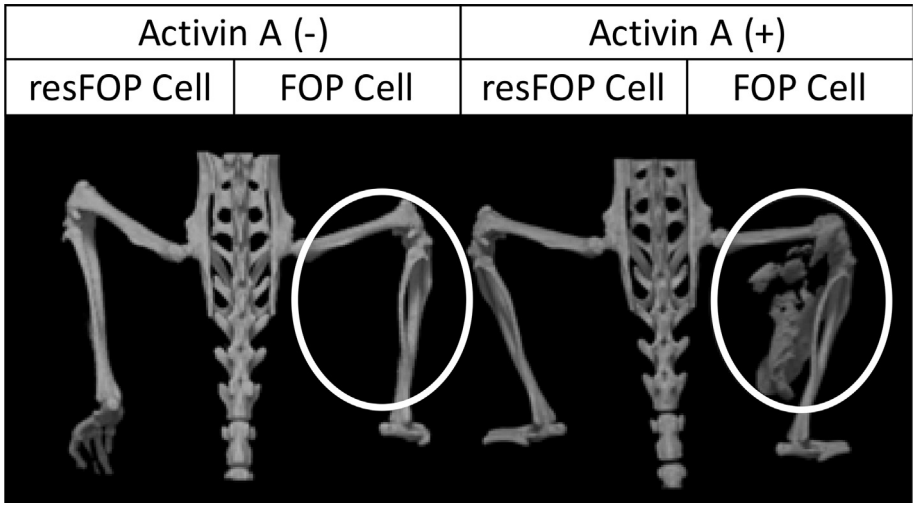

Fig. 2. Ectopic bones are formed following FOP-MSC transplantation. μCT images of formed ectopic bone in mice. FOP-MSCs (right leg) and resFOP-MSCs (left leg) were transplanted into the gastrocnemius muscle of mice. White circles show the transplanted area. This figure has been modified from Hino et al. [70].

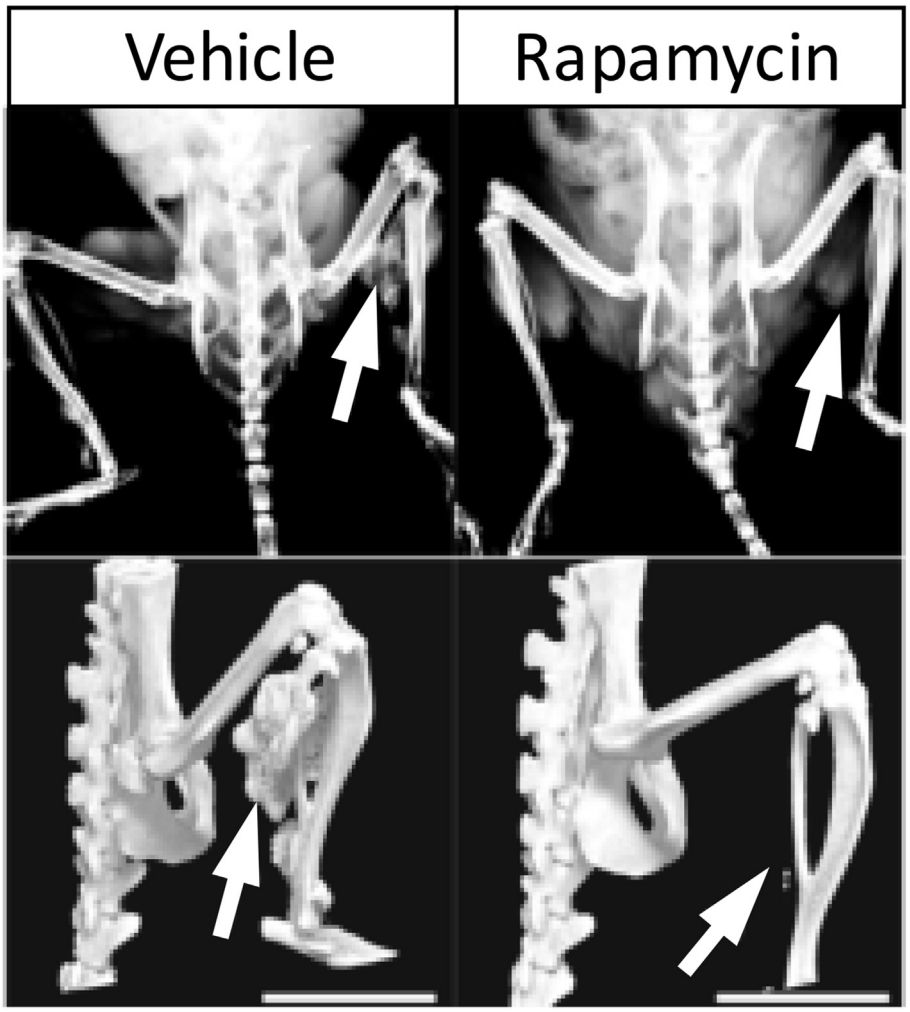

Fig. 3. Rapamycin suppresses ectopic bone formation. X-ray (upper row) and μCT (bottom row) images. Administration of rapamycin suppressed activin A-triggered ectopic bone derived from FOP-MSCs. White arrows show the transplanted area. This figure has been modified from Hino et al. [77].

suppressed the ectopic ossification in multiple mouse models, including FOP-ACVR1 transgenic mice and ectopic ossification model mice utilizing FOP-iPSCs. We also revealed that one of the hit compounds, TAK 165, acts on mTOR indirectly, unlike rapamycin; this indicates the possibility of mTOR signaling dysfunction as a contributing factor in FOP and its possible application to the drug as well. Although clinical trials using rapamycin have been in progress, there is a substantial reason for considering a new drug, besides rapamycin, since all patients do not have the same phenotype. Moreover, it may be conceivable to achieve greater effects by combining multiple drugs.

3. Conclusion

Rapamycin, an mTOR inhibitor, has already been used in Japan as a drug for lymphangioleiomyomatosis. This concept of extended application to the other disease is called drug repositioning. This drug repositioning helps in reducing the cost and time of drug development as compared to the canonical drug discovery approaches [81]. The tag-team "iPSCs-technology based drug discovery × drug repositioning" has great potential to accelerate the process of discovering new therapeutic agents. In recent years, this tag-team has revealed several drug candidates for diseases other than FOP. For example, it is reported that statins, already being used as therapeutic agents for hypercholesterolemia, can be applied to the treatment for achondroplasia (bone lineage disorder) and tanatophoric dysplasia as well [82]. In addition, another group reported that bosutinib, an anticancer agent used for treating chronic myelogenous leukemia, can also be used to treat amyotrophic lateral sclerosis, a type of neurodegenerative disease [83]. Moreover, it is demonstrated that rapamycin would be effective for the treatment of Pendred syndrome, a hereditary disorder typically associated with hearing loss [84].

Although rapamycin is expected to suppress the formation of new ectopic bone in FOP cases, it cannot be effective for already formed ectopic bone. In this regard, it is indispensable to develop new drugs or treatments that can remove existing ectopic bone tissue. Or adopting new therapeutic approaches such as genome-editing for FOP treatment could be possible in future. Also, several type of non-classic mutations that result in phenotypic variations in terms of the severity and onset of disease are reported but previous report have mainly used patient-derived iPSCs harboring a classic FOP mutation, R206H. Thus, further progress in research is still desired.

Study approval

All experiments 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.

Conflicts of interest

All authors declare no conflict of interest.

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