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Original Article

Impairment of the transition from proliferative stage to prehypertrophic stage in chondrogenic differentiation of human induced pluripotent stem cells harboring the causative mutation of achondroplasia in fibroblast growth factor receptor 3

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ABSTRACT

Introduction: Achondroplasia (ACH) is a congenital disease which causes dwarfism and many symptoms resulting from skeletal dysplasia. Because present therapeutic strategies are mainly surgical procedures as symptomatic treatments, development of a radical treatment is desired. Clarification of the ACH pathology is essential for creating a new remedy. However, there are many questions about the disease mechanisms that have not been answered.
Methods: As a single base substitution of the *FGFR3* gene had been proved to be the ACH causing genome mutation, our group established disease specific iPS cells by introducing the causative mutation of achondroplasia into human iPS cells by CRISPR/Cas9 based genome editing. These cells were differentiated towards chondrocytes, then the gene and protein expressions were examined by real time RT-PCR and Western blotting, respectively.
Results: Based on the western blotting analysis, the FGFR3 protein and phosphorylated ERK were increased in the *FGFR3* mutated iPS cells compared to the control cells, while the *FGFR3* gene expression was suppressed in the *FGFR3* mutated iPS cells. According to chondrogenic differentiation experiments, the *IHH* expression level was increased in the control cells as the differentiation progressed. On the other hand, up-regulation of the *IHH* gene expression was suppressed in the *FGFR3* mutated iPS cells.
Conclusions: These results suggested that chondrocyte maturation was impaired between the proliferative stage and prehypertrophic stage in the chondrocytes of ACH. The development of chemical compounds which affect the specific maturation stage of chondrocytes is expected to contribute to the ACH treatment, and *FGFR3* genome-edited hiPSCs will be a valuable tool in such research studies.

1. Introduction

Achondroplasia (ACH) is the most common form of human dwarfism and its incidence is between one in 10,000 and one in 30,000 live births [1,2]. Bone forming deficiency attributed to repressed endochondral ossification causes many symptoms such as hydrocephalus, spinal cord compression, otitis media, and misalignment of teeth [1]. Although it is known that gain-of-function mutation of the *FGFR3* gene causes ACH, it is still controversial as to how the mutation for the receptor stimulates the downstream signal. For example, it was reported that inhibition of the receptor internalization causes hyperactivation of the FGFR3 signals [2,3]. According to other reports, disruption of

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ubiquitylation retards the receptor degradation in lysosomes [2,4]. Besides, some groups established ACH model mice and confirmed a narrowed hypertrophic zone in the growth plates of the mice [5]. However, there are few reports which clearly estimated the molecular mechanisms of ACH by applying human specimens. Current therapeutic strategies for ACH are mainly surgical treatments, e.g., surgical limb lengthening for short stature, ventricular shunts for hydrocephalus, and decompression surgery for spinal cord compression. These are not radical treatments, but symptomatic treatments, and surgeons sometimes have to choose highly-invasive treatments for patients at an early age depending on the clinical condition. Meanwhile, there are many reports about pharmacotherapy such as growth hormone, C-type natriuretic peptide (CNP) analog and statin. Although they may have potential to radically improve the symptoms of ACH, the effectiveness or safety of these agents has not yet been established [6–8]. Therefore, more research for ACH pathogenesis is required to discover novel treatment strategies.

The induced pluripotent stem cell (iPS cell) has a differentiation potency towards the tridermic cell [9]. In addition to its application to regenerative medicine, the iPS cell is applicable in pathology and drug discovery research [8,10]. By differentiating disease specific iPS cells established from patients with certain diseases, cells or tissues affected by the diseases can be obtained and utilized for the determination of disease mechanisms. These cells can be also used for the reactivity assessment of cells toward therapeutic agents. In spite of the usefulness of patient specific iPS cells, the difficulty to obtain patient cells hinders their use.

Recent advancements in genome editing techniques, such as the clustered regularly interspaced palindromic repeat (CRISPR)/Cas9 system and the transcription activator-like effector nucleases (TALEN) system, have made it possible to remove, insert or mutate genes as intended. This means that, even in the case of rare diseases, of which samples are hard to obtain, disease specific cells can be established by genome editing, as long as the disease causing gene is already known. It is said that the *FGFR3* gene (1138G→A) mutation is most commonly observed in ACH [11,12]. Therefore, in this report, we established human iPS cells harboring a homozygous mutation in the *FGFR3* gene (1138G→A) by using the genome editing technique, CRISPR/Cas9 system. By differentiating the cells with a mutation toward the chondrocytes, we showed that the mutation caused accumulation of the FGFR3, hyperactivity of ERK signals and impairment of the chondrogenic maturation. These cells established in the present study will become useful tools for the analyses of the disease mechanisms and drug discovery for ACH.

2. Material and methods

2.1. Establishment of FGFR3 genome-edited hiPSCs

gRNA plasmid DNA and Oligo DNA were designed to introduce the 1138G→A mutation to the *FGFR3* gene of human iPS cells (HPS0002 253G1; RIKEN BRC) [13] by CRISPR/Cas9 genome editing. To determine the gRNA, the Cas9 activity to cleave the DNA in transfected cells by electroporation, genome DNA from iPS cells introduced with the gRNA plasmid and the Cas9 expression plasmid by electroporator NEPA21 (NEPAGENE), were examined by a T7 Endonuclease I (T7E1) assay. The gRNA plasmid, Oligo DNA and Cas9 expression plasmid were introduced into the hiPS cells by electroporation (Table 1). The transfected cells had been seeded on a culture dish, then colonies were harvested and *FGFR3* direct sequencing was performed to confirm whether the 1138G→A mutation had been integrated. Established genome-edited iPS cells were proliferated and stocked for subsequent experiments. (Outsourced to ReproCELL, Inc., and Takara Bio, Inc.)

2.2. hiPS cell culture and chondrogenic differentiation

Genome-edited hiPSCs were differentiated towards chondrocytes by the methods reported by Oldershaw et al. with some modifications [14].

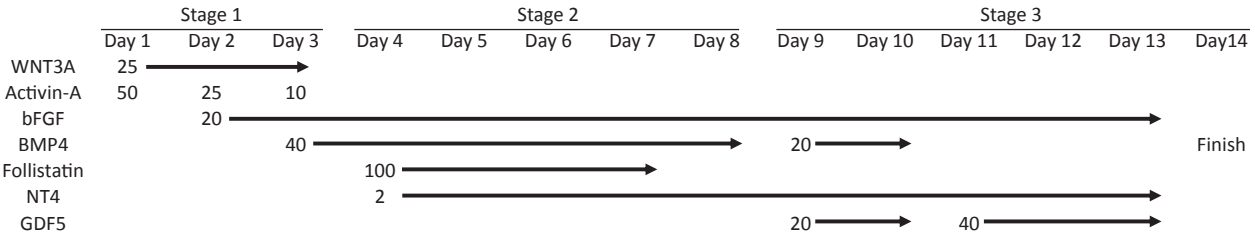
The hiPSCs were initially cultured in a proliferating medium containing DMEM F12 Gluta Max (Gibco), 100 UNIT/ml penicillin and 100 μg/ml streptomycin (Sigma), 20% (vol/vol) knockout serum replacement (KSR; Gibco), 1% (vol/vol) nonessential amino acids (Gibco), 55 μM 2-mercaptoethanol (Sigma), and 10 ng/ml FGF-2 (KAKEN) on feeder cells. As the feeder cells for the iPS cells, murine embryonic fibroblasts (MEF) were harvested from embryos of ICR mice which were 12 days pregnant (CLEA Japan), and initially treated with mitomycin-C (Sigma). The iPSCs were dissociated from the dish by CTK solution using a cell scraper and seeded on a feeder or feeder-free cell culture dish. The medium was changed every day and the frequency of passage was basically twice a week. The CTK solution was used as the cell dissociation reagent. The CTK solution's composition was 0.25% (wt/vol) trypsin (BD), 1 mg/ml Collagenase type IV (Gibco), 20% (vol/vol) KSR (Gibco), and 1 mM $CaCl_2$ (Wako) in PBS. The feeder-free culture dish was coated by hES Qualified Matrigel (Corning), and STEM PRO hESC SFM (Invitrogen) was used as the medium. To refine the iPSCs in the feeder-free state, the iPSCs were passaged more than 3 times on the feeder-free environment. When the iPS cell's proliferating area had reached over 80% confluence of the growth area of the cell culture dish (about 10 days after the day when the last passage had been performed), differentiation of the iPSCs toward the chondrocytes was started. The chondrogenic differentiation was completed by the 14-day medium changes. The chondrogenic differentiation medium was composed of a basal medium and cytokines.

The basal medium was composed of DMEM F12 Gluta Max (Gibco), 1% (vol/vol) ITS (Sigma), 10% (vol/vol) B27 supplement (Gibco), 1% (vol/vol) non-essential amino acid (Gibco), 50 UNIT/ml penicillin, 50 μg/ml streptomycin (Sigma), and 89 μM 2-mercaptoethanol (Gibco), and implemented with cytokines such as WNT-3A (Pepro Tech), Activin-A (Pepro Tech), bFGF (Pepro Tech), BMP-4 (Pepro Tech), Follistatin (Pepro Tech), GDF5 (PRO SPEC) and NT4 (Pepro Tech) as shown in Table 2. As previously reported, we assumed Stage 1 cells (at day 4 from when differentiation had been started on day 1) as the primitive streak–mesendoderm cells, Stage 2 cells (day 9) as the mesoderm cells, and Stage 3 cells (day 14) as the chondrocytes.

Table 1
Setting for iPSCs electroporation by NEPA21.

Setting value											
Poring pulse						Transfer pulse					
Voltage (V)	Pulse width (ms)	Pulse interval (ms)	Frequency	Decay rate (%)	Polar character	Voltage (V)	Pulse width (ms)	Pulse interval (ms)	Frequency	Decay rate (%)	Polar character
125	5	50	2	10	+	20	50	50	5	40	+/−

Table 2
Cytokine composition in chondrogenic differentiation medium on each days; Unit: ng/ml.



2.3. mRNA collection and real time RT-PCR assay

The mRNA was extracted from pluripotent hiPSCs at the end of Stage 1 (day 4), Stage 2 (day 9), and Stage 3 (day 14) by Isogen (NIPPON GENE). The expressions of *FGFR3*, *COL2A1*, *IHH* and *GAPDH* were examined by real time RT-PCR. The mRNA had been reversed transcribed into cording DNA (cDNA) by a PrimeScript RT-PCR kit (Perfect Real Time) and the cDNA was used for the PCR to prepare copy number standards of each gene. Subsequently, the real time RT-PCR was performed using cDNA with the Fast SYBR Green Maser Mix (Applied Biosystems). The gene expression was normalized by the *GAPDH* expression. All the primer sequences are shown in Table 3.

2.4. Protein extraction and Western blotting

At differentiation stage 3 (day 14), the protein was extracted by m-PER (Thermo) according to the manufacturer's instruction. The protein had been separated on a PVDF membrane by SDS-PAGE, and anti-FGFR3 (abcam; ab137084), anti-p-ERK (Cell Signaling; #4370), anti-ERK (Cell Signaling; #4695) and anti-ACTIN (Sigma; A2066) antibodies were applied for the detection of each factor. Furthermore, the density of each protein band was quantified by ImageJ (National Institutes of Health) and the quantified protein expression was normalized by dividing the FGFR3 and p-ERK levels by the ACTIN and ERK levels, respectively.

3. Results

3.1. Establishment of FGFR3 genome-edited hiPSCs

The gRNA and Oligo DNA were designed to introduce 1138G→A (G380R, ACH causing mutation) to the human *FGFR3* gene (NM_000142, Fig. 1a). To confirm the activity of the gRNA and Cas9 to cleave the DNA in transfected cells, the T7 endonuclease I assay (T7E1 assay) was performed. The gRNA denoted in Fig. 1a with Cas9 were introduced to the hiPSCs by electroporation, and the genomic DNAs were analyzed by electrophoresis (Fig. 1b). The DNA fragments generated by cleavage were found in the sample treated with gRNA, Cas9 and T7E1, indicating their activity. To establish the hiPSCs with the ACH causing mutation, the hiPSCs were co-transfected with gRNA, Oligo DNA and Cas9. Ninety-five transfected cell clones were obtained. An *FGFR3* direct sequencing was conducted for each clone, and finally 2 cell clones (lot #52, #68 cells) were confirmed to be introduced with the 1138G→A *FGFR3* mutation (Fig. 1c).

Table 3
Primer sequences; *FGFR3*, *COL2A1*, *IHH*, *GAPDH*.

FGFR3	F	GCACACCCTACGTTACCGTG
	R	GCCTCGTCAGCCTCCACCAG
COL2A1	F	GGCAATAGCAGGTTCACGTACA
	R	CGATAACAGTCTTGCCCCACTT
IHH	F	TCAGCGATGTGCTCATTTTC
	R	ACCAGTGTCCCATGCTTTGT
GAPDH	F	ATGGGGAAGGTGAAGGTCG
	R	TAAAAGCAGCCCTGGTGACC

3.2. Profiles of genome-edited hiPSCs

To exclude the effects of the genome editing procedures on the cell characteristics, cells, which underwent the same procedure but no mutation was introduced [Mutation (−); lot #60], were used as the control cells. By phase contrast microscopic observation, there were no obvious differences in morphology between the control cells (lot #60) and Mutation (+) cells (lot #68 and #52) (Fig. 2a and data not shown). Although each cell line originated from a single colony, there would be the possibility that the colony consisted of more than 1 clone, and cells without mutation became dominant in the clones. To confirm that cells still bore the mutation, lots #52 and #68 cells adapted in the feeder-free environment were analyzed by DNA sequencing for the *FGFR3* gene (Fig. 2b; Outsourced to Takara Bio, Inc.). The Lot #52 and #68 cells under the feeder-free condition still had a single base substitution mutation in the *FGFR3*.

3.3. Analysis of FGFR3 and downstream signal

The ACH mutation is reported to cause accumulation of the FGFR3 proteins [4]. To confirm this was also the case with the cells established by us; the genome-edited hiPSCs were differentiated toward the chondrocytes. At the end of differentiation stage 3 (day 14; chondrocyte state), proteins were obtained and analyzed for FGFR3, ACTIN, p-ERK and ERK by Western blotting (Fig. 3a). The FGFR3 and p-ERK levels in the Mutation (+) cells were higher than those in the Mutation (−) cells, as previously reported [4,8]. mRNA was also extracted from the pluripotent hiPSCs, at the end of Stage 1 (day 4), Stage 2 (day 9), and Stage 3 (day 14), and analyzed for the expression of *FGFR3* by real time RT-PCR (Fig. 3b). In the Mutation (−) cells, the expression of *FGFR3* increased as the chondrogenic maturation. On the other hand, *FGFR3* was down-regulated in stage 3 of the Mutation (+) cells, as is the case in the thanatophoric dysplasia type I (TD1) cells [8]. The *FGFR3* expression may be suppressed by the negative feedback system as a result of protein accumulation of the mutated FGFR3, as mentioned in a previous report [8].

3.4. Analysis of chondrogenic markers

To examine the consequences of the 1138G→A mutation in the human *FGFR3* gene, the early chondrogenic marker gene, *COL2A1*, and late chondrogenic marker gene, *IHH*, were analyzed by real time RT-PCR using the same mRNA as in Fig. 3b (Fig. 4). *COL2A1* was

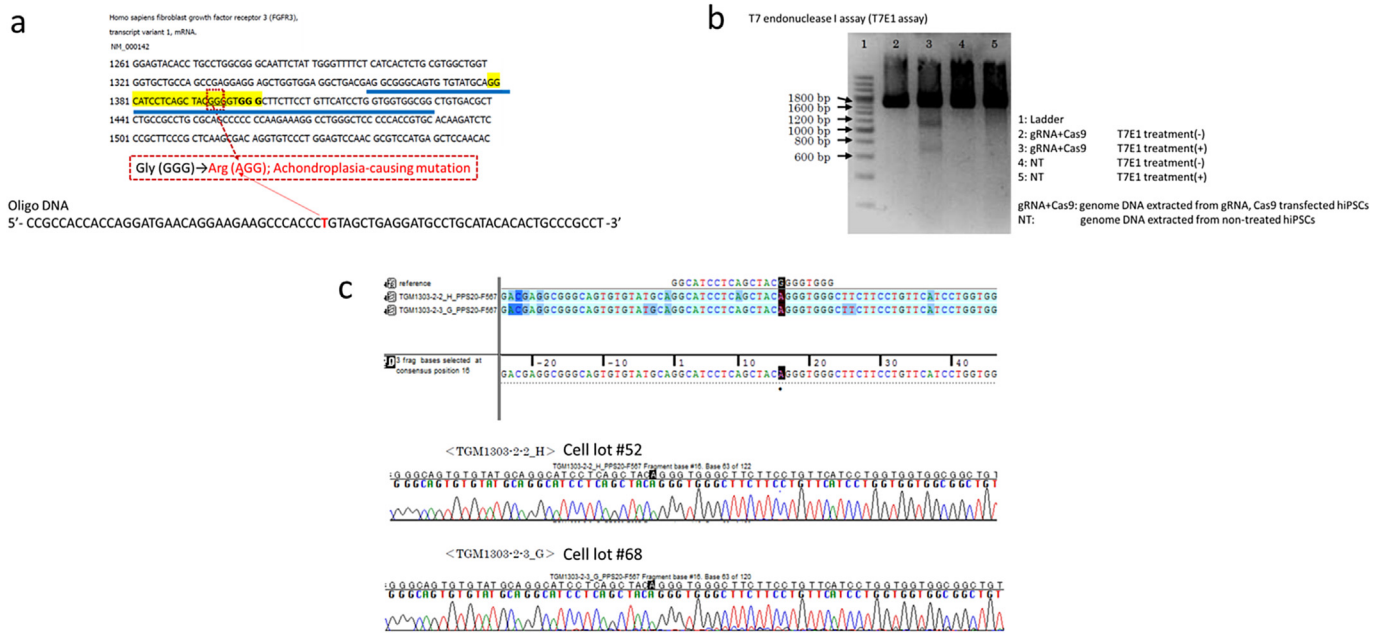


Fig. 1. hiPSCs *FGFR3* genome editing. (a) Designs for gRNA and Oligo DNA; gRNA target site (yellow marker), point mutation preferred site (red dotted frame), Oligo DNA sequence and complementary RNA sequence (blue underline). (b) T7 endonuclease I assay (T7E1 assay); assay for determining gRNA, Cas9 action to cleave the DNA in transfected cells by electroporation. (c) *FGFR3* direct sequence data of lots #52 and #68 cells; sequences nearby point mutation preferred site (shaded region) are shown.

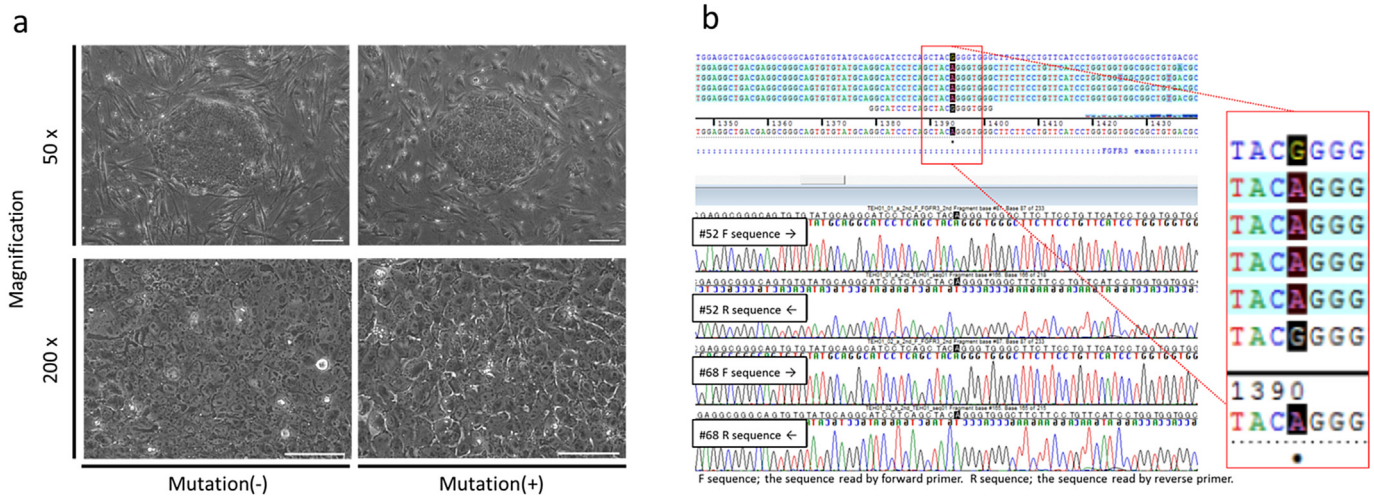


Fig. 2. Genome-edited hiPSCs profiles. (a) Phase contrast microscopic images; genome-edited hiPSCs in which *FGFR3* mutation was accurately introduced [Mutation (+)]; lot #68 cells and genome-edited hiPSCs in which *FGFR3* mutation was not introduced [*FGFR3* sequence was normal; Mutation (−)]; lot #60 cells are shown. Lots #60 and #68 cells were in the pluripotent state and on the mouse embryonic fibroblast (MEF) feeder cells. Scale bars = 200 μm in 50 × figures, 100 μm in 200 × figures. (b) Sequence data of lots #52 and #68 cells adapted in feeder-free environment; sequences nearby point mutation preferred site (shaded region) are shown and extended (red frame).

up-regulated in both the Mutation (−) and Mutation (+) cells. On the other hand, an increase in the *IHH* expression level observed in Stage 3 of the Mutation (−) cells was suppressed in the Mutation (+) cells. This finding seemingly corresponds to the shortening of the hypertrophic zones observed in the growth plate of the achondroplasia model mice [5].

4. Discussion

The *FGFR3* genome-edited hiPSCs were established by the CRISPR/Cas9 system in this study. Except for the CRISPR/Cas9 system, there are also the TALEN system and zinc finger nuclease (ZFN) system for genome editing. While these two systems require designing binding proteins for each DNA target site, the CRISPR/Cas9 system requires only a modification in the guide RNA target sequence [15,16]. Although the CRISPR/Cas9 system is easier to apply, this system may be more likely to cause off-target mutations as some groups claim [17]. On the other hand, a comparable specificity of the CRISPR/Cas9 system with the TALEN system is reported when they are used for human iPS cells [18]. In our experiments, the phenotype of the genome-edited hiPSCs, e.g., the protein expression was consistent with that of the ACH cells in previous reports [4,8], and besides, they resembled the human iPS cell morphology regarding the shapes of the control cells and FGFR3 mutated cells in the microscopic images shown in Fig. 2a. Accordingly, we considered that the *FGFR3* genome-edited

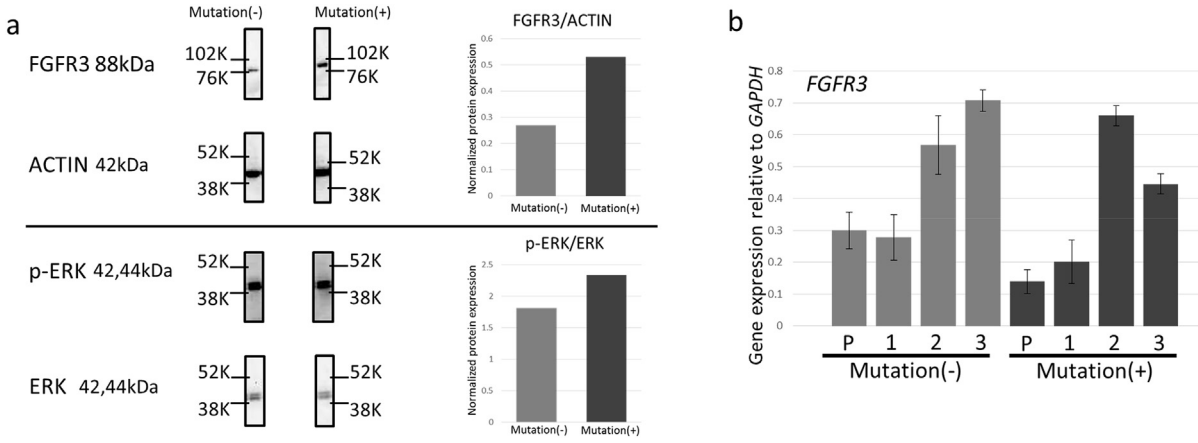


Fig. 3. Analysis of FGFR3 and downstream signal. (a) Protein expressions of FGFR3, ACTIN, p-ERK, ERK from differentiating hiPSCs at the end of Stage 3 were analyzed by Western blotting. Genome-edited hiPSCs in which *FGFR3* mutation was accurately introduced [Mutation (+)]; lot #68 cells and genome-edited hiPSCs in which *FGFR3* mutation was not introduced [*FGFR3* sequence was normal; Mutation (−)]; lot #60 cells were used. Density of bands were measured by ImageJ and normalized through dividing FGFR3 and p-ERK level by ACTIN and ERK level, respectively. (b) Gene expression level of *FGFR3* was analyzed by Real time RT-PCR in mRNA from pluripotent hiPSCs (P), at the end of Stage 1 (1), Stage 2 (2), and Stage 3 (3). Mutation (+); lot #68 cells and Mutation (−); lot #60 cells were used. Gene expression was calculated on copy number and normalized by *GAPDH* expression. Error bars; mean ± S.D.

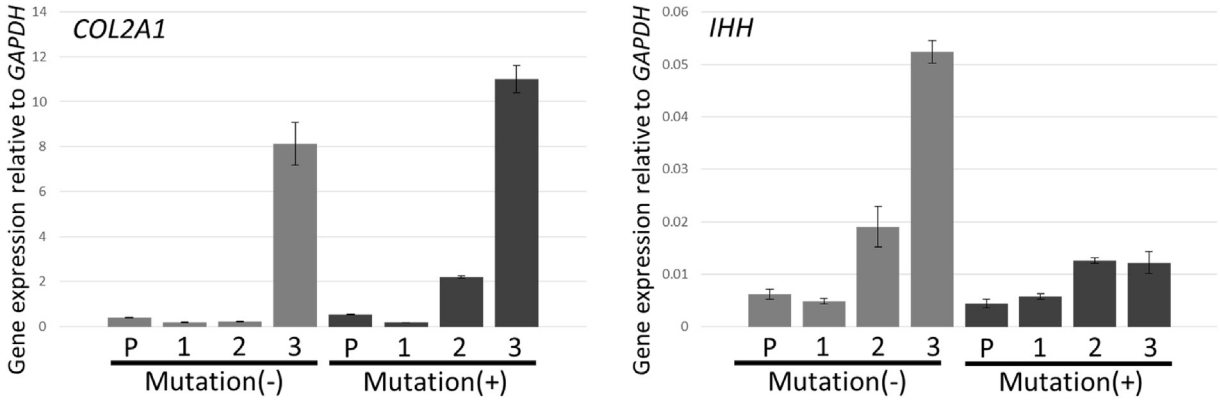


Fig. 4. Analysis of chondrogenic markers. Gene expression levels of *COL2A1*, *IHH* were analyzed by Real time RT-PCR in mRNA from pluripotent hiPSCs (P), at the end of Stage 1 (1), Stage 2 (2), and Stage 3 (3). Genome-edited hiPSCs in which *FGFR3* mutation was accurately introduced [Mutation (+)]; lot #68 cells and genome-edited hiPSCs in which *FGFR3* mutation was not introduced [*FGFR3* sequence was normal; Mutation (−)]; lot #60 cells were used. Gene expression was calculated on copy number and normalized by *GAPDH* expression. Error bars; mean ± S.D.

hiPSCs, which we established, may have no critical mutations in the off-target sites. To add credibility to the experiments, the whole-genome sequence or whole-exome sequence may be performed.

As shown in Fig. 3, the FGFR3 protein expression level increased in the *FGFR3* mutated cells in differentiation stage 3, while the *FGFR3* gene expression level decreased in the same cells. These findings are consistent with those reported for the thanatophoric dysplasia which is also caused by the gain-of-function mutation of the *FGFR3* gene and has worse phenotypes [8]. This suggests that the *FGFR3* gene expression is controlled by the negative feedback system [8].

Recently, Yamashita et al. reported the ACH pathology and drug discovery research using iPS cells [8]. They differentiated iPS cells established from ACH patients toward chondrocytes and analyzed them to show the availability of statin as a therapeutic agent for the ACH. On the other hand, our group established the *FGFR3* genome-edited hiPSCs by the CRISPR/Cas9 system. We compared the genome-edited hiPSCs in which the 1138G→A *FGFR3* mutation was introduced; Mutation (+) cells and the *FGFR3* mutation were not introduced; Mutation (−) was the control cells. Because the cellular origins of these cells were the same in the experimental group and control group, and they underwent the same treatments, a precise analysis could be performed.

In the differentiation stage 3, the *COL2* gene expression level increased in both groups. On the other hand, the *IHH* gene expression in the *FGFR3* mutated cells was lower than that in the control cells. As the *IHH* gene is expressed from the prehypertrophic stage [19,20], our data suggested that maturation of the mutated cells into the prehypertrophic chondrocytes was impaired.

Therefore, it was assumed that one of the main systems of the ACH pathological mechanisms was impairment of the transiting proliferative stage to the prehypertrophic stage of the chondrocytes. In previous studies, shortening of the hypertrophic zone in ACH model mice had been reported [5], and accordingly, it was suggested that our established cells might reflect the ACH disease phenotypes. However, compared to the heterozygous mutation, which was most common in the ACH patients [7], in this research, the *FGFR3* mutated cells were introduced by homozygous mutation. Since the homozygous ACH would cause early death in humans [1], it had to be considered that the hiPS cells harboring a homozygous

mutation in the *FGFR3* gene might exhibit more severe phenotypes than the ACH patient's pathological conditions.

As a future plan for applying the *FGFR3* genome-edited hiPSCs, we considered that these cells could be used for research to develop ACH treatment agents. In particular, we are initially planning to structure a high throughput screening system to select drug candidate compounds which have potencies to up-regulate the *IHH* gene expression in *FGFR3* mutated cells at chondrogenic differentiation stage 3. If such effective compounds are discovered by the screening system, each compound will be precisely confirmed by another in-vitro assay and animal experiments. When the data from these experiments are collected and sufficiently considered, we can address the clinical research.

5. Conclusions

In summary, we have established achondroplasia disease specific iPS cells by genome editing. One possibility was shown that the ACH disease phenotypes would appear from the proliferative stage to prehypertrophic stage by differentiating the genome-edited hiPSCs towards chondrocytes and analyzing those cells. The *FGFR3* genome-edited iPS cells we established will become a powerful tool for the analysis of molecular mechanisms of ACH and the development of a pharmacological agent which selectively affects the chondrocytes in a stage-specific manner.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.reth.2016.11.002.

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