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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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.

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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
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 iPSC cells introduced with the gRNA plasmid and the Cas9 expression plasmid by electroporator NEPA21 (NEPAGENE), were examined by a T7 Endonuclease 1 (T7E1) assay. The gRNA plasmid, Oligo DNA and Cas9 expression plasmid were introduced into the hiPSC 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. hiPSC 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 iPScells 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’s composition was 0.25% wt/vol trypsin (BD), 1 mg/ml Collagenase type IV (Gibco), 20% (vol/vol) KSR (Gibco), and 1 mM CaCl2 (Wako) in PBS. The feeder-free culture dish was coated by hES Qualified Matrigel (Corning), and STEM PRO hiESC SFM (Invitrogen) was used as the medium. To refine the iPS cells in the feeder-free state, the iPS cells 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 iPS cells 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) nonessential 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—mesodermoid 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. |
|---------------------------------------------|
| **Voltage (V)** | **Pulse width (ms)** | **Pulse interval (ms)** | **Frequency** | **Decay rate (%)** | **Polar character** |
|-----------------|----------------------|-------------------------|--------------|-------------------|-------------------|
| Porung pulse    |                      |                         |              |                   |                   |
| 125             | 5                    | 50                      | 2            | 10                | +                 |
| Transfer pulse  |                      |                         |              |                   |                   |
| 20              | 50                   | 50                      | 5            | 40                | +/-               |
Furthermore, the density of each protein band was quantified. A2066) antibodies were applied for the detection of each factor. #4370), anti-ERK (Cell Signaling; #4695) and anti-ACTIN (Sigma; #A2228) antibodies were used for Western blotting. The protein had been separated on a PVDF membrane by SDS-PAGE, and anti-FGFR3 (abcam; ab137084), anti-p-ERK (Cell Signaling; #4370) antibodies were applied for the detection of each factor. Subsequently, the real time RT-PCR was performed using cDNA with the Fast SYBR Green Master Mix (Applied Biosystems). The gene expression was normalized by dividing the FGFR3 and p-ERK levels by the ACTIN and ERK levels, respectively.

### 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. 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 to cleave the DNA in transfected cells, the T7 endonuclease I assay was performed. The gRNA denoted in Fig. 1a with Cas9 to cleave the DNA in transfected cells, the T7 endonuclease I assay was performed. The gRNA denoted in Fig. 1a with Cas9 to cleave the DNA in transfected cells, the T7 endonuclease I assay was performed.

### 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 chondrocyte. 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 1 (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
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 hiPSCs...
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 iPSCs 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 iPSCs 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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