Targeted gene correction and functional recovery in achondroplasia patient-derived iPSCs

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Achondroplasia (ACH) is the most common genetic form of dwarfism and belongs to dominant monogenic disorder caused by a gain-of-function point mutation in the transmembrane region of *FGFR3*. Stem cells and gene-editing technology provide us with effective methods and ideas for research and treatment of ACH. Here we generated non-integrated iPSCs from one ACH girl's skin and one ACH boy's urine via Sendai virus. We found that the chondrogenic differentiation ability of ACH iPSCs was confined compared with healthy iPSCs. When the mutation of ACH iPSCs from skin and urine was precisely corrected by CRISPR-Cas9, the chondrogenic differentiation ability of them could be restored. Furthermore, these corrected iPSCs displayed pluripotency, maintained normal karyotype, and did not demonstrate off-target indels. Our study may provide important theoretical and experimental basis for the stem cell research and treatment of ACH.

Achondroplasia (ACH), the most common genetic form of short-limb dwarfism, is autosomal dominant monogenic disorder (MGD) caused by a gain-of-function point mutation in the transmembrane region of fibroblast growth factor receptor 3 (*FGFR3*). There are currently two mutation sites reported - Gly380Arg and Gly375Cys, the former occupies a vast majority of ACH patients.[1,2] Because homozygous ACH patients have a much more severe phenotype and rarely survive,[3,4] most of the ACH patients seen are heterozygous mutations. The estimated frequency of ACH is 1 in 25,000 (Male 1/15000), with at least 80% of the cases being sporadic.[5] Like many

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other MGDs, there are no effective therapeutic methods for ACH even though the mutation has been found.[1] Fortunately, stem cell research provides new hope for potential ACH treatments. Patient-specific stem cells can aid scientists in the investigation of specific molecular mechanisms and can be used to screen for the discovery of new drugs.[6,7] In addition, with powerful genome editing tools used, such as clustered regulatory interspaced short palindromic repeat (CRISPR)-Cas9 system,[8] mutation can be corrected in ACH stem cells *in vitro*. Through screening and safety assessment in animal models *in vivo*, stem cell transplantation therapy may provide an effective and novel treatment method.[6]

In this study, we isolated and cultured skin, urine, and white adipose-derived somatic cells from three ACH patients of Gly380Arg mutation. Further we generated non-integrated iPSC lines from one ACH girl's skin and one ACH boy's urine by Sendai virus. However adipose-derived mesenchymal stem cells (AD-MSCs) could not be reprogrammed to iPSCs. We found the chondrogenic differentiation ability of ACH iPSCs was confined compared with that of healthy iPSCs. When the mutation of ACH iPSCs from skin and urine was precisely corrected by CRISPR-Cas9, the chondrogenic differentiation ability of them was restored. Via cell immunofluorescence, Alkaline Phosphatase (AP) Staining, karyotyping and sequencing analysis, we found these corrected iPSCs continued to keep pluripotency, maintained normal chromosomal number and structure, and didn't display off-target. Our study may provide important theoretical and experimental basis for the stem cell research and treatment of ACH.

Results

Identification of ACH Patients and Isolation and Culture of Somatic Cells. We recruited three ACH patient donors, including an 8-year-old girl, a 7-year-old boy and one 37-year-old adult man. Genetic identification was confirmed by DNA genome sequencing, and the donors are all heterozygous mutations of a G-A transition at nucleotide 1138 of the cDNA of *FGFR3* (Figure 1a). In addition, their clinical phenotypes are consistent with the NIH-defined characteristics, such as short arms and legs, enlarged head, and prominent forehead. We punched skin tissue and collected urine from the two children to culture skin fibroblasts (SFs) and urine-derived cells (UCs). Via liposuction, we obtained adipose tissue from the adult man to culture AD-MSCs. After the small pieces of skins were placed and cultured in the dish for 2 weeks, SFs gradually climbed out to begin to proliferate (Figure 1b and c). After urine-derived pellets were cultured for one week, UCs formed colonies and showed epithelial cell morphology (Figure 1d and e). After SVF from adipose tissue was seeded and cultured for 2-3 days, AD-MSCs grew out and exhibited a typical fibroblast-like morphology (Figure 1f and g).

Generation of Non-Integrated iPSCs from ACH Patients. To generate non-integrated iPSCs from ACH patients, we used Sendai virus to transfect cells. Three weeks after transduction of SFs and UCs, lots of ES cell-like colonies appeared (Figure 2a and b). After picking up and expanding single colony, we established non-integrated iPSC lines from girl's skin (GF) and boy's urine (BU) (Figure 2c and d). These iPSCs expressed pluripotent protein - NANOG, OCT4, SOX2, SSEA-4, and TRA1-60, while didn't express SSEA-1 (Figure 2g). They also showed AP positive (Figure 2h). In addition, karyotyping analysis indicated that they maintained normal chromosomal number and structure (Figure 2i). However, it was surprised that iPSCs could not be generated from AD-MSCs. Although small and unhealthy colonies could appear (Figure 2e), they gradually became apoptotic and eventually died (Figure 2f).

Chondrogenic Differentiation Capability of ACH iPSCs. To figure out whether the mutations of ACH iPSCs from skin and urine would affect their function, we performed detection of their chondrogenic differentiation capability. We used healthy human iPSCs and ACH iPSCs side by side to induce chondrogenic differentiation in a multi-step method (Figure 3a). We found that the chondrogenic clusters from ACH iPSCs were less and smaller than those from healthy iPSCs (Figure 3b). By Safranin O staining, we discovered the cartilage tissue derived from ACH iPSCs showed less and weaker positive areas and lower cartilage density than that from healthy iPSCs (Figure 3c). These results indicated that the cartilage tissue derived from ACH iPSCs could produce less and thinner cartilaginous extracellular matrix. RT-qPCR results also exhibited that cartilage tissue derived from ACH iPSCs expressed lower chondrocyte-specific genes - SOX9, COL2A1, and ACAN (Figure 3d). Our results suggested the chondrogenic differentiation ability of ACH iPSCs was confined compared with that of healthy iPSCs.

Gene Correction of ACH iPSCs by CRISPR-Cas9. To design sgRNAs, we input 64 bp DNA sequences of ACH *FGFR3* around the point mutation site into Guide Design Resouces. More than 10 sgRNAs were generated and we selected the sg2RNA of highest score for use (Figure 4a). To correct the point mutation in ACH iPSCs, we used 131 nucleotides ssODN donor as the homology arm which contained the point mutation site (Figure 4a). 24-48 hours after CRISPR-sgRNAs and ssODNs were transected into ACH iPSCs from skin and urine, we detected the transfection efficiency by FACS. The RFP positive ratio was 3.6% (Figure 4b). By sequencing analysis of more than 100 RFP positive single cell colonies from skin and more than 100 RFP positive single cell colonies from urine, we found one completely corrected cell line from skin and one completely corrected cell line from urine (Figure 4c), and the total efficiency was less than 1%. We analyzed the two corrected iPSC lines and found they expressed pluripotent protein -

NANOG, OCT4, SOX2, SSEA-4 and TRA1-60, while didn't express SSEA-1 (Figure 4d). They also indicated AP positive (Figure 4e). The karyotyping analysis verified they maintained normal chromosomal number and structure (Figure 4f). Sequencing analysis of potential offtarget sites given by Guide Design Resouces showed that no off-target indels were identified.

Functional Recovery of Corrected ACH iPSCs. To detect whether the function of corrected ACH iPSCs from skin and urine was restored, we performed the detection of chondrogenic differentiation ability of them. Through morphological observation, we found that chondrogenic clusters from corrected ACH iPSCs obviously increased compared with those from uncorrected cells (Figure 5a). Via Safranin O staining, we found there were more and stronger positive areas in cartilage tissue from corrected ACH iPSCs than that from uncorrected cells (Figure 5b). EdU cell proliferation assay displayed corrected cells possessed higher positive radio than uncorrected cells (Figure 5c). Compared with cartilage tissue from ACH iPSCs, RT-qPCR results also revealed cartilage tissue from corrected ACH iPSCs expressed higher chondrocyte-specific genes - SOX9, COL2A1, and ACAN (Figure 5d). These results suggested the chondrogenic differentiation ability of corrected ACH iPSCs was restored.

Discussion

Whenever about 25,000 children are born, one of them may be an ACH patient.[5] For ACH patients, not only will they lose a lot of opportunities to get education and job because of their short stature, but they also have many complications, such as hydrocephalus, obesity and sleep apnea etc. The older they are, the more serious they will be, which will seriously affect their longevity. The average life expectancy for this cohort was decreased by 15 years compared with the US population.[5] Moreover, the social concern of this disease is very low. At present there are no effective therapeutic methods for ACH. With the rapid development of stem cell biology and gene-editing technology, the research and treatment of ACH not only show promising, but also provide ideas and experimental basis for the investigation of other MGDs.[6]

In this study we first collected and isolated three different tissue samples from ACH patients to culture somatic cells, including the most commonly used skin, the most easily available urine, and a large amount of acquired adipose tissue that can produce multipotent MSCs with chondrogenic differentiation ability. We found that, like skin cells, ACH patient urine-derived cells could be efficiently reprogrammed into iPSCs, which may provide new donor cells for research of MGDs. However, to our surprise, ACH patient-derived AD-MSCs could not be reprogrammed into iPSCs. In fact, previously reported study[9,10] and our unpublished results found that healthy human AD-MSCs could be reprogrammed more efficiently to iPSCs than skin cells. Given ACH is a

regeneration dysfunction disorder of MSC-derived chondrocyte caused by *FGFR3* mutation, our results suggested that perhaps the point mutation affected the reprogramming ability of AD-MSCs.

Our initial hypothesis was that point mutations might affect the chondrogenic differentiation ability of ACH iPSCs. Indeed, our experimental results confirmed chondrogenic differentiation ability of ACH iPSCs was confined compared with that of healthy iPSCs. When we used CRISPR-Cas9 to correct point mutation of ACH iPSCs, we obtained two completely corrected cell lines (one from skin, another from urine) from more than two hundred single cell colonies. The efficiency of precise homology directed repair (HDR) was less than 1%. Fortunately, we found these two completely corrected iPSC lines still displayed pluripotency and maintained normal karyotype. Sequencing analysis of potential offtarget sites suggested no off-target indels were identified. Finally, we detected whether the function of corrected ACH iPSC could be improved. Via chondrogenic differentiation, EdU cell proliferation assay, and RT-qPCR experiments, we found their chondrogenic differentiation ability was indeed restored compared with uncorrected cells.

In summary, our study results of ACH iPSCs *in vitro* provided a solid and important foundation for the further exploration of ACH research and treatment *in vivo*. At present, we are constructing point mutation mouse model of ACH. Next, we will transplant the corrected ACH patient-derived MSCs or chondrocyte precursor cells into the mice to verify their function *in vivo* and explore the effect of this cell replacement therapy to ACH. Although challenges remain, the clinical application of patient-specific stem cells will be pursued through further advances in basic research (Figure 6).

Materials and Methods

Research subjects and ethical statement. Research subjects in this study included two children and one adult man. Skin and urine samples were obtained from the children. Liposuction was performed on the adult man. All human subject protocols were reviewed and approved by the Ethical Review Board of the Renji Hospital, Shanghai. All subjects gave signed informed consent.

Isolation and culture of SFs. Skin biopsies were performed by using a sterile 3 mm skin punch from the locally anesthetized lower legs. The skin tissue was cut in smaller pieces, placed in a 6-well plate and allowed to grow for 2 weeks in fibroblast medium (DMEM, 10% fetal bovine serum (FBS), penicillin/streptomycin (P/S) and glutamine). Above regents were all from ThermoFisher.

Culture of UCs. The culture of UCs followed the method established by Pei laboratory.[11] Briefly, urine samples were collected into 50 ml tube and were centrifuged at 400 g for 10 minutes. The supernatants were aspirated carefully, and about 1 ml volume at the bottom was kept in the tube.

The remaining was resuspended by washing buffer (PBS containing amphotericin B and P/S) and centrifuged again. After discarding supernatants, the pellets were suspended by 1 ml primary medium. Then the cell suspensions were seeded into 12-well plates coated with gelatin and cultured in cell incubator. The primary medium contained DMEM/Ham's F-12 1:1 (ThermoFisher), 10% FBS, REGM Renal Epithelial Cell Growth Medium SingleQuots Kit (Lonza), amphotericin B (Selleck) and P/S. After 4 days, the medium was carefully changed to proliferation medium (Lonza REBM BulletKit).

Isolation and culture of AD-MSCs. Adipose biopsy was obtained by liposuction. Isolation and culture of AD-MSCs followed our previous reports.[12] Briefly, adipose tissue was digested with 0.1% type I collagenase (Sigma) in PBS solution on a shaker at 37°C for 1 hour. Cell suspensions were then centrifuged to obtain stromal vascular fraction (SVF). The SVF was suspended and cultured in fibroblast medium.

PCR and Sanger Sequencing. Total genomic DNA was extracted from patient peripheral blood, SFs, UCs, AD-MSCs, and iPSCs by using the Genomic DNA Extraction Kit (TaKaRa). PCR was performed by using Q5 High-Fidelity DNA Polymerase (New England Biolabs, NEB). *FGFR3* primers were

Forward: 5'-AGGAGCTGGTGGAGGCTGA-3',

Reverse: 5'-GGAGATCTTGTGCACGGTGG-3'.

PCR reactions were then purified by using GeneJET Gel Extraction Kit (ThermoFisher) and sequenced by the Genewiz.

Generation of non-integrated iPSCs. Induction of iPSCs from SFs, UCs, and AD-MSCs was performed by using CytoTune iPS 2.0 Sendai Reprogramming Kit (ThermoFisher). Briefly, approximately 200,000 donor cells were seeded in a 6-well plate to culture. Two days later, cells were transduced with the Reprogramming Kit. 24 hours after transduction, the medium was replaced with fresh medium every other day. On day 8 after transduction, the donor cells were trypsinized and 5000 cells were seeded into a 6-well plate. The media were replaced by E8 (StemCell) every day thereafter. 3-4 weeks after transduction, single cell colonies were picked for expansion and characterization.

Chondrogenic differentiation of iPSCs. This method referred to report of Tsumaki laboratory (Yamashita et al., 2014). After iPSCs formed high-density cell colonies in E8, the medium was changed to initial chondrogenic medium, including DMEM/F12 (ThermoFisher), 10 ng/ml of Wnt3A (R&D), 10 ng/ml of Activin A (Peprotech), 1% ITS (ThermoFisher), and 1% FBS

(ThermoFisher). On day 3, the medium was changed to the basal medium, including DMEM (ThermoFisher), 1% ITS, 1% FBS, 2 mM L-glutamine, non-essential amino acids, 1mM Napyruvate (ThermoFisher), 50 μg/ml ascorbic acid (Sigma), 10 ng/ml BMP2 (Peprotech), 10 ng/ml TGFβ1 (Peprotech), and 10 ng/ml GDF5 (Peprotech). 10 ng/ml bFGF (Peprotech) was added to the chondrogenic medium from day 3 to day 14. After Day 42, the medium was changed to fibroblast medium.

AP staining. AP staining was performed by using Vector Blue AP Substrate Kit (Vector). Procedure was conducted in accordance with the instruction.

Design of single guide RNAs (sgRNAs) and construction of vector. sgRNAs were designed to target point mutation site by using the Guide Design Resouces of Zhang lab (https://zlab.bio/guide-design-resources). Then oligo sgRNAs were annealed and ligated to the pSpCas9(BB)-2A-RFP plasmid which was digested with Bbs I (NEB) enzyme.

Transfection of CRISPR into iPSCs. One million iPSCs were resuspended in 800 μl cold PBS and mixed with targeting and single-stranded oligo DNA nucleotides (ssODNs) donor (5 μg CRISPR targeting plasmid and 40 μg donor). The cells were electroporated using the Human Stem Cell Nucleofector Kit 2 (Lonza) on Nucleofector 2b Device (Lonza). Cells were recovered and re-plated on plates with ROCK inhibitor. 24-48 hours after electroporation, about 5000 RFP positive cells were sorted and seeded into 100 mm plate by BD FACS Aria II. One week later, single colony was pick up and expanded for sequencing analysis.

Off-target effect analysis. 18 potential “off-target” sites given by Guide Design Resouces were identified. DNA sequencing of PCR products amplified from these sites was performed.

Cell immunofluorescence. iPSCs were fixed in 4% paraformaldehyde solution for 15 minutes at room temperature (RT). Next, they were permeabilized by using 0.1% Triton X-100 in PBS for 20 minutes at RT. After they were blocked for 1 hour in 5% goat serum, the cells were incubated with primary antibodies Nanog (Abcam), SSEA-1 (Millipore), Oct4 (Cell signal), SSEA-4 (Millipore), Sox2 (Abcam), and TRA-1-60 (Abcam) overnight at 4°C. The cells were incubated with a fluorescently coupled secondary antibody for 1 hour at RT, meanwhile the nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI; Sigma). Images were captured on a Leica confocal microscope.

Karyotyping. The iPSCs were cultured in E8 with 0.1 μg/ml colchicine (ThermoFisher) for 2 hours. After medium being removed, they were incubated in 0.56% potassium chloride for 40 minutes,

then fixed in methanol: acetic acid (3:1 in volume) overnight. The cell suspensions were dropped onto cool slides and stained with Giemsa (ThermoFisher) for 15 minutes. Finally, chromosome analysis was performed under the karyotyping image analysis system. More than 15 metaphase spreads of each sample were analyzed.

RNA extraction and RT-qPCR. Total RNAs were extracted from whole cells using TRIzol RNA Isolation Reagents (Thermofisher). cDNA was synthesized from total RNA with a Hifair II 1st Strand cDNA Synthesis Kit (Yeasen) for qPCR (TB Green Premix Ex Taq kit, TakaRa). PCR procedure was carried out according to the kit instructions. Primers used were as follows:

SOX9 F: AGACCTTTGGGCTGCCTTAT
R: TAGCCTCCCTCACTCCAAGA
COL2A1 F: TTTCCCAGGTCAAGATGGTC
R: CTTCAGCACCTGTCTCACCA
ACAN F: AGGCAGCGTGATCCTTACC
R: GGCCTCTCCAGTCTCATTCTC
β-ACTIN F: TGGCACCACACCTTCTACAATGAGC
R: GCACAGCTTCTCCTTAATGTCACGC

EdU Staining. We used BeyoClick EdU-594 kit (Beyotime) for staining. Briefly, cells were incubated for 24 hours in 10 μM EdU medium at 37ºC in incubator. After EdU solution was removed, the cells were washed twice in PBS. Then they were fixed and performed immunocytochemistry procedure.

Safranin O staining. Safranin O staining Kit (ScienCell Research Laboratories, Inc. #8384) was used to perform this experiment. Procedure was in accordance with the instruction.

Conflicts of Interest

There is no potential conflict of interest.

Acknowledgements

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Author contributions

ZH, GMF, LY, and LF performed experiments and analyzed data. WWY provided experimental platform and financial support; QYR conceived and designed the study, performed experiments, analyzed data, provided financial support and wrote the manuscript.

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Figures and figure legends

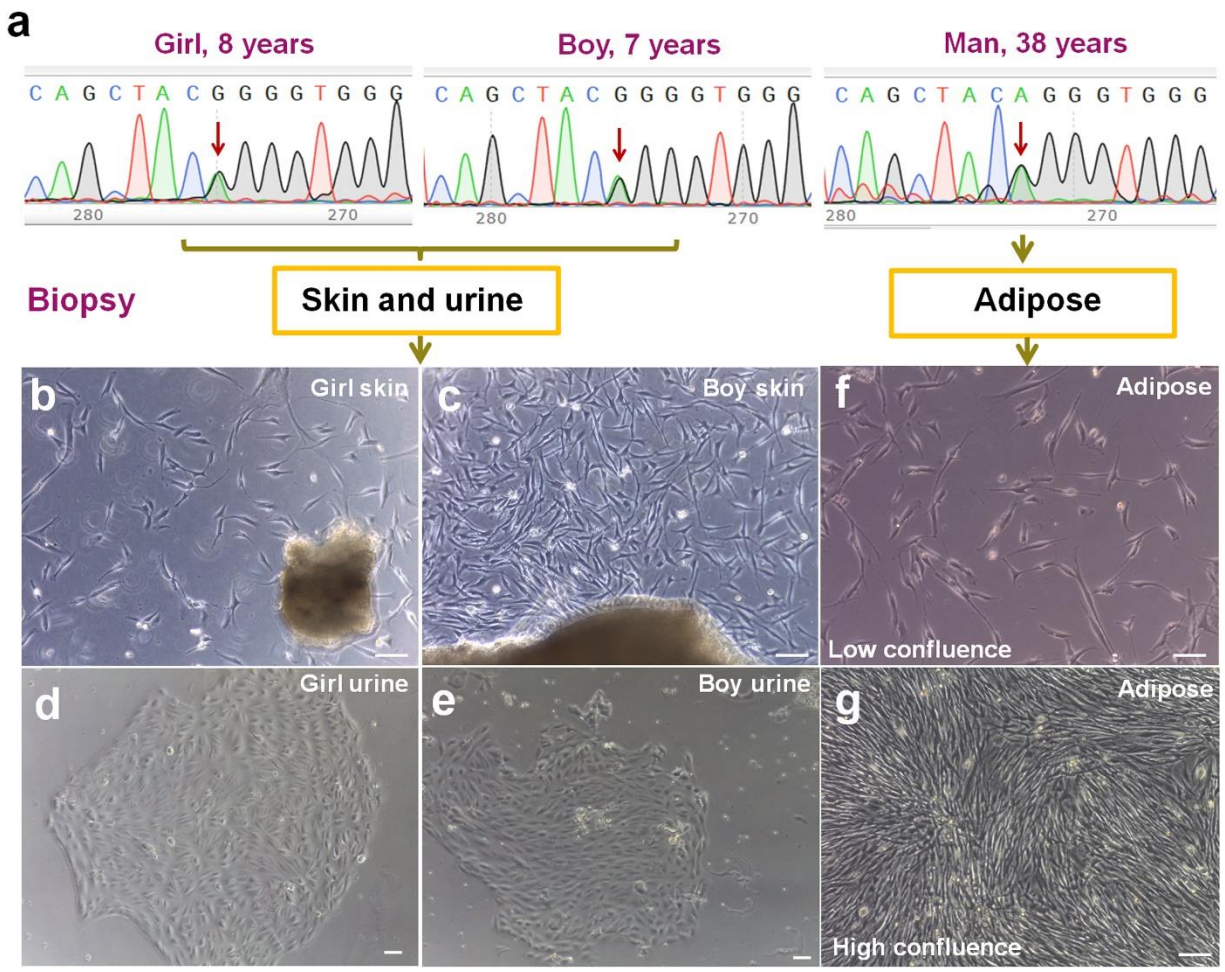

Figure 1 Identification of ACH patients and isolation and culture of somatic cells. **(a)** Sequence of three ACH patient donors showed they are all heterozygous mutation of a G-A transition at nucleotide 1138 of the cDNA. **(b, c)** SFs from girl and boy. **(d, e)** UC colonies from girl and boy showed epithelial cell morphology. **(f, g)** AD-MSCs from adult man exhibited a typical fibroblast-like morphology. Bar in all panel: 10 μm.

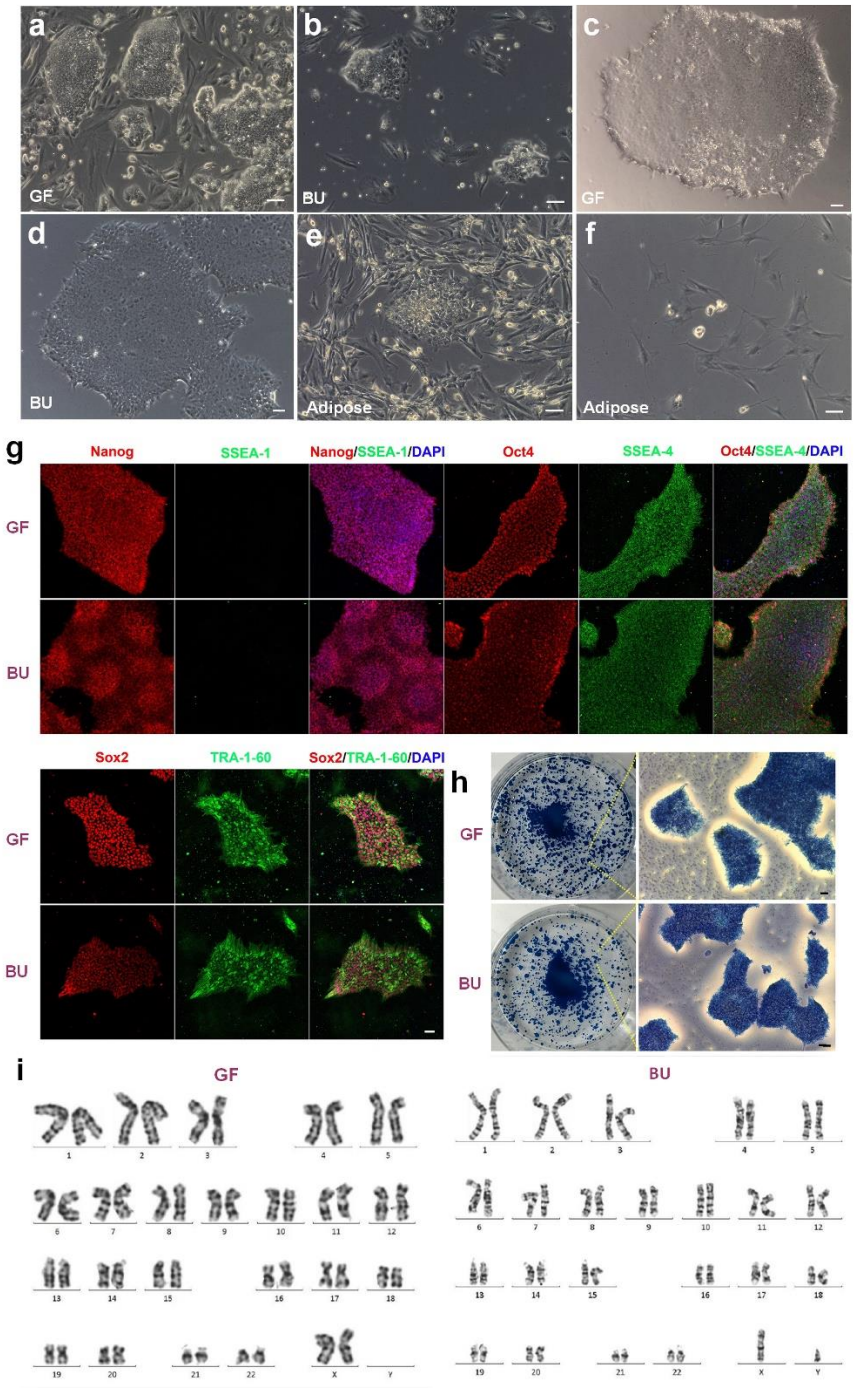

Figure 2 Generation of non-integrated iPSCs from ACH patients and characterization of them. **(a, b)** 3 weeks ES cell-like colonies from girl SFs (GF) and boy UCs (BU) after transfection. **(c)** Expanded iPSCs from GF. **(d)** Expanded iPSCs from BU. **(e)** 3 weeks ES cell-like colonies from AD-MSCs after transfection, they were small and unhealthy. **(f)** Colonies from AD-MSCs gradually became apoptotic and eventually died. **(g)** iPSCs from GF and BU expressed pluripotent protein - NANOG, OCT4, SOX2, SSEA4 and TRA1-60, but didn't express SSEA-1. **(h)** iPSCs from GF and BU showed AP positive. **(i)** iPSCs from GF and BU indicated normal chromosomal number and structure. Bar in all panel: 10 μm.

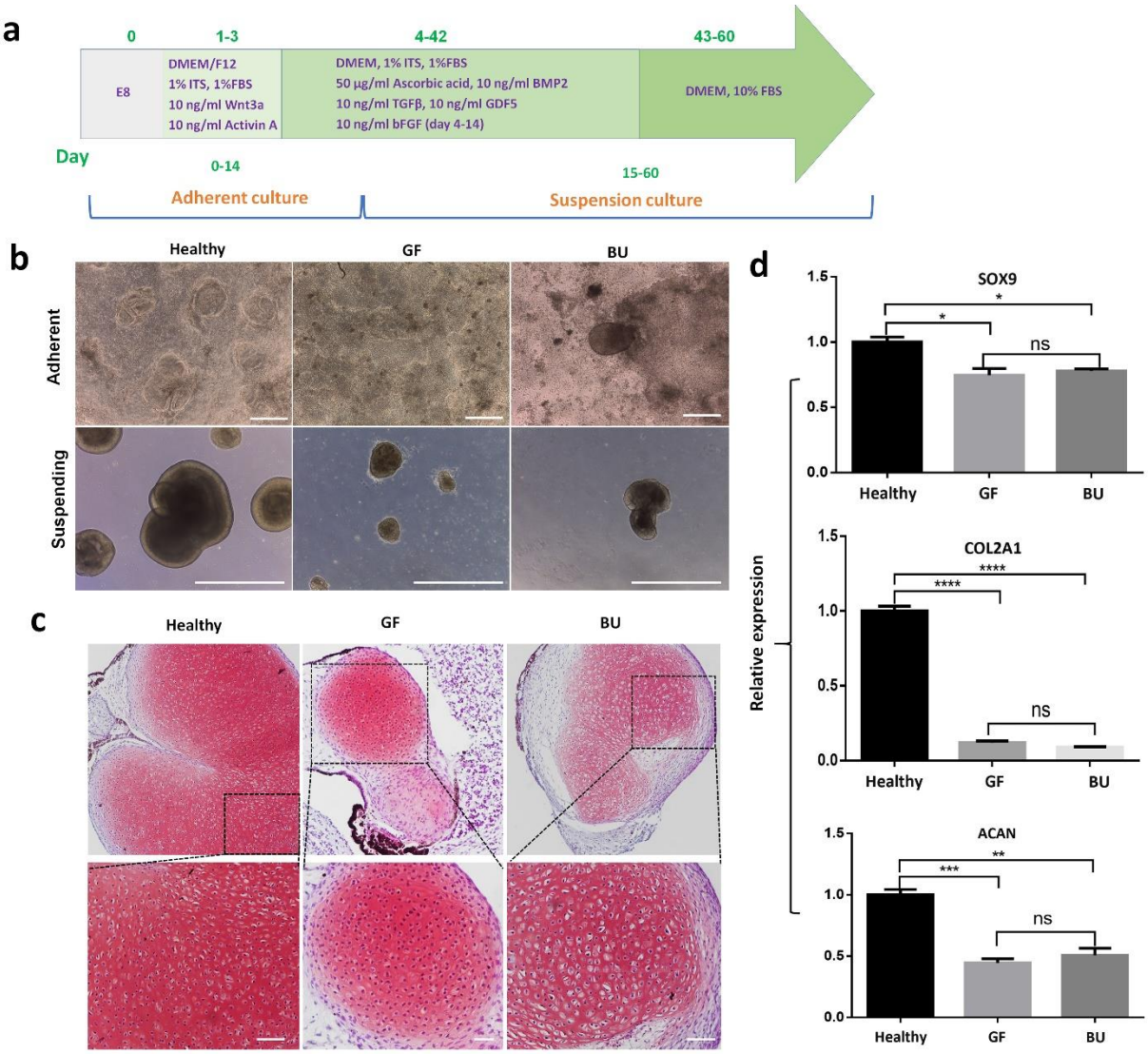

Figure 3 Chondrogenic differentiation of ACH iPSCs. **(a)** A multi-step chondrogenic differentiation method. **(b)** Chondrogenic clusters from ACH iPSCs were less and smaller than those from healthy iPSCs. Bar: 100 μm. **(c)** Safranin O staining displayed there were less and weaker positive areas in cartilage tissue from ACH iPSCs than that from healthy iPSCs. Bar: 10 μm**. (d)** RT-qPCR results also exhibited that cartilage tissue derived from ACH iPSCs expressed lower chondrocyte-specific genes SOX9, COL2A1, and ACAN.

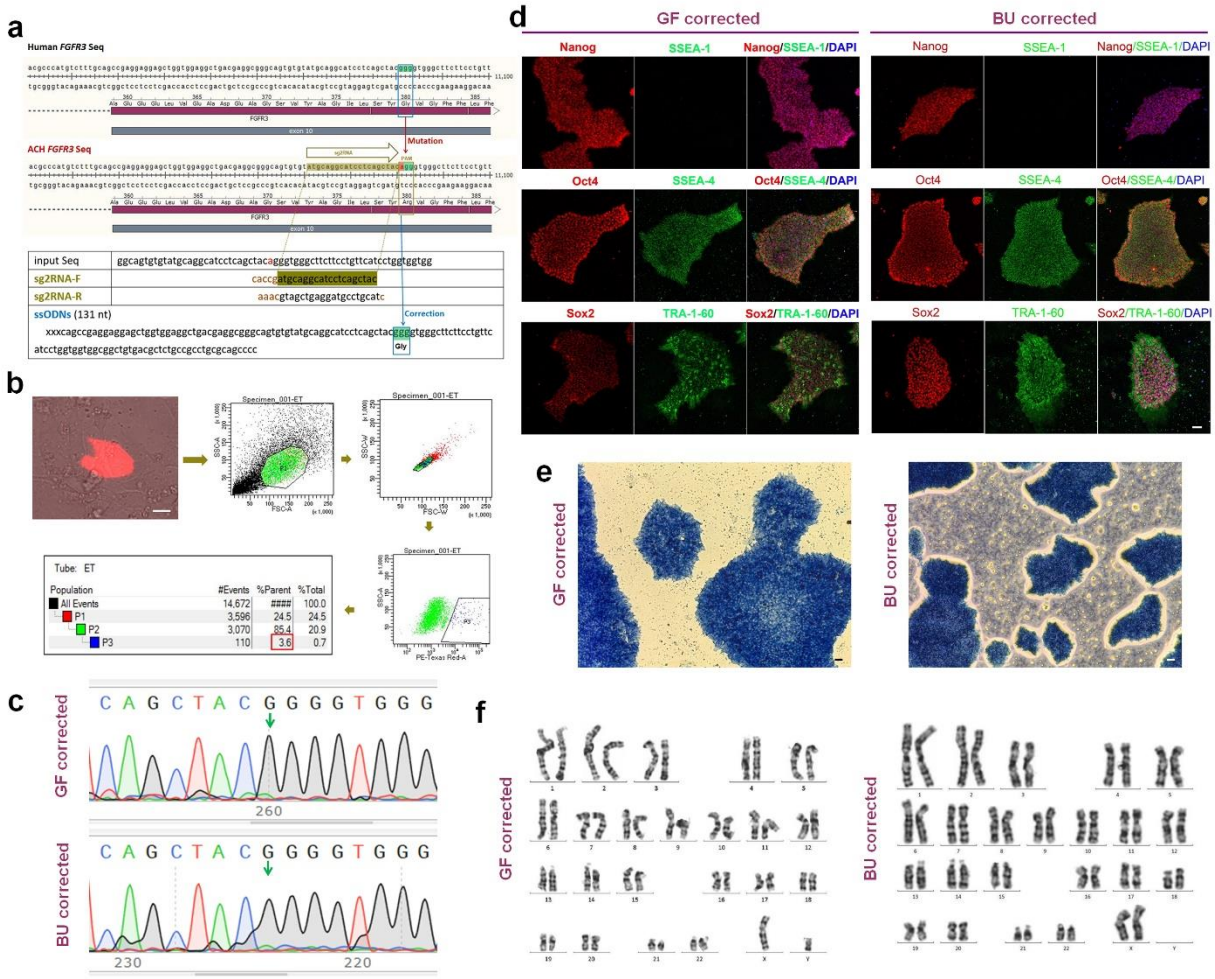

Figure 4 Gene correction of ACH iPSCs by CRISPR-Cas9 and characterization of corrected iPSCs. **(a)** Designed sgRNA and ssODNs around the point mutation site. **(b)** FACS detection showed the RFP positive cells were 3.6%. **(c)** Two corrected iPSC lines showed normal DNA sequence. **(d)** Corrected iPSCs expressed NANOG, OCT4, SOX2, SSEA4 and TRA1-60, but didn't express SSEA-1. **(e)** Corrected iPSCs indicated AP positive. **(f)** Corrected iPSCs showed normal chromosomal number and structure. Bar in all panel: 10 μm.

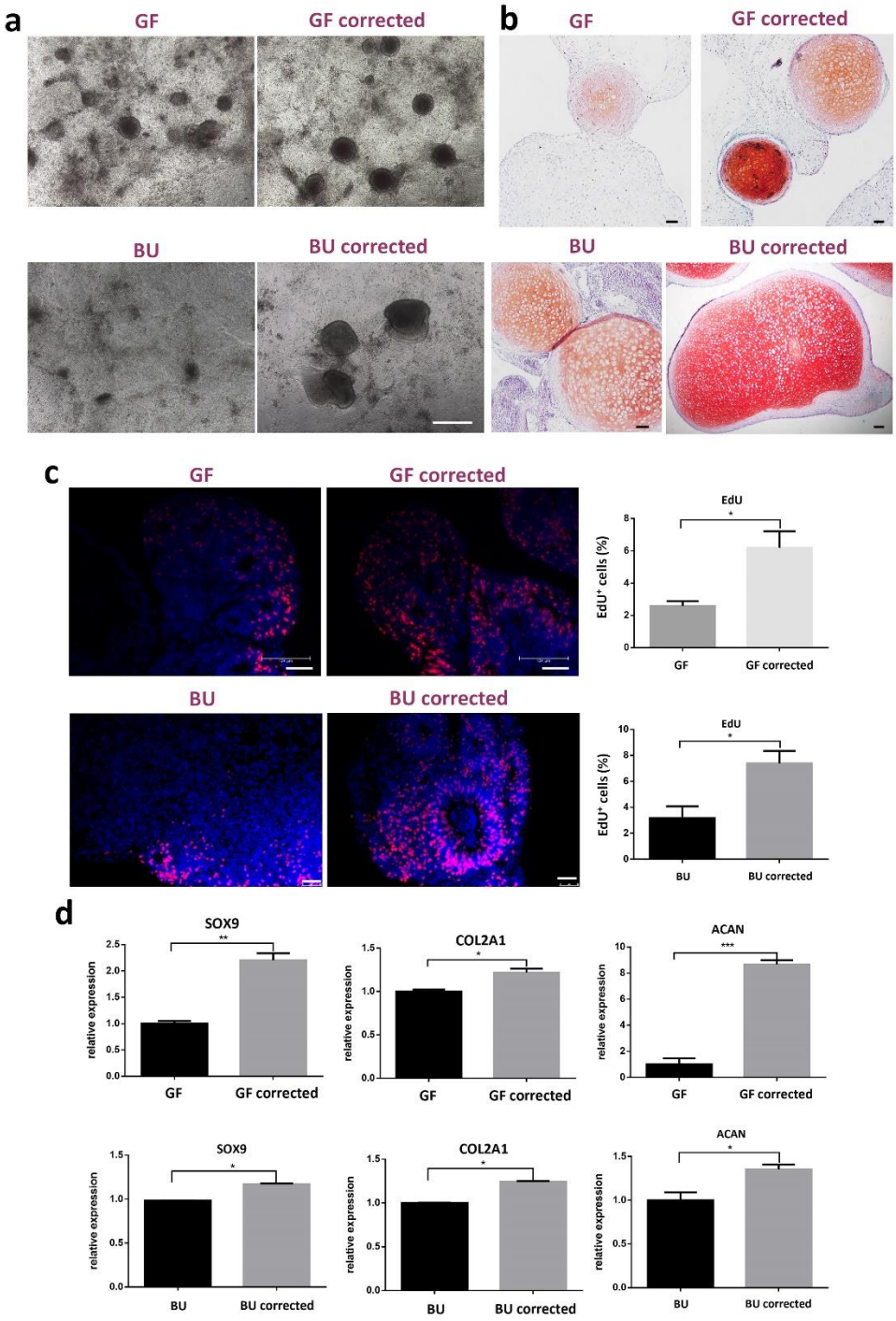

Figure 5 Chondrogenic differentiation of corrected ACH iPSCs. **(a)** Chondrogenic clusters from corrected iPSCs obviously increased compared with the uncorrected cells. Bar: 100 μm. **(b)** Safranin O staining illustrated cartilage tissue from corrected ACH iPSCs demonstrated more and stronger positive areas, and higher cartilage density than uncorrected cells. Bar: 10 μm. **(c)** EdU cell proliferation assay indicated corrected iPSCs showed higher positive radio than uncorrected cells. Bar: 50 μm. **(d)** RT-qPCR results also revealed that the expression level of chondrocyte-specific genes - SOX9, COL2A1, and ACAN from corrected ACH iPSCs were higher than that of ACH iPSCs.

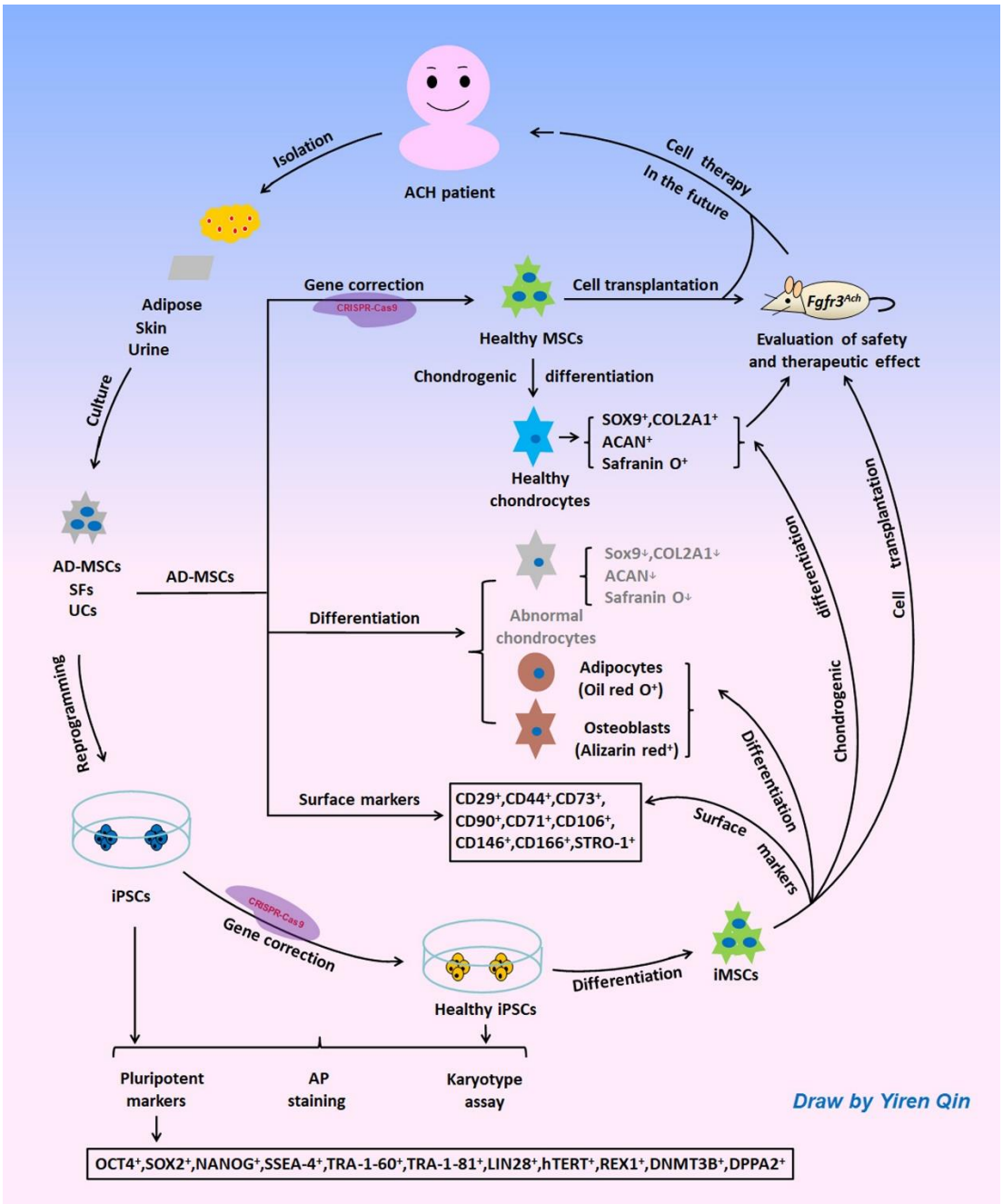

Figure 6 Diagrammatic strategy of ACH patient-derived stem cell research. Somatic cells can be isolated and cultured from ACH adipose, skin, and urine, and further can be reprogrammed into iPSCs. After gene correction of iPSCs or AD-MSCs via CRISPR-Cas9, they can differentiate into healthy cells, such as MSCs or chondrocyte precursor cells. Then these healthy cells can be transplanted into ACH mouse model to assess their relative safety and therapeutic effects.