Generation of non-integrated iPSCs from achondroplasia patient skin and urine and gene correction in them using CRISPR-Cas9

Yiren Qin[1,2,*]

[1]Interdisciplinary Research Center on Biology and Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai 201210, China.

[2]State Key Laboratory of Oncogenes and Related Genes, Renji-Med X Clinical Stem Cell Research Center, Ren Ji Hospital, School of Medicine and School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200127, China.

[*]Corresponding authors. Email: yirenqin@hotmail.com

Abstract

Achondroplasia (ACH) is the most common genetic form of dwarfism and belong to dominant monogenetic 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 the vast majority of ACH patients. Homozygous ACH patients have a much more severe phenotype and rarely survive, so most of the ACH patients seen are heterozygous mutations. Like many other MGDs, there are no effective therapeutic methods for ACH even though the cause of the mutation has been found. With the rapid development of stem cell biology and gene-editing technology, the research of ACH is not only important, but also provides theoretical and experimental basis for the investigation of other MGDs. In this study, we generated non-integrated induced pluripotent stem cell (iPSC) lines from ACH patient skin and urine of Gly380Arg mutation. Via powerful gene-editing tool - CRISPR-Cas9, we successfully obtained 2 completely corrected knock-in cell lines. They not only expressed pluripotent markers, maintained normal chromosomal number and structure, but also no off-target indels were identified. This is not only the first time to obtain ACH iPSCs using the most readily available biopsy sample - urine, the first to obtain non-integrated ACH iPSCs, but also is the first to perform gene correction in ACH research. Our study may provide important theoretical and experimental basis for the next step of stem cell research and treatment of ACH in the future.

Key words: achondroplasia, iPSCs, gene correction, CRISPR-Cas9

Introduction

Achondroplasia (ACH), the most common genetic form of short-limb dwarfism, is autosomal dominant monogenetic 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 the vast majority of ACH patients, a G-A transition, at nucleotide 1138 of the cDNA (Shiang et al. 1994; Superti-Furga et al., 1995). Because homozygous ACH patients have a much more severe phenotype and rarely survive (Pauli et al., 1983; Hecht et al., 1986), 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 (Horton et al., 2007). Like many other MGDs, there are no effective therapeutic methods for ACH even though the cause of the mutation has been found (Qin and Gao, 2016). Fortunately, stem cell research provides new hope for potential ACH therapies. The generation of patient-specific stem cells, which can aid scientists in the investigation of specific molecular mechanisms and can be used to screen for the discovery of new drugs (Qin and Gao, 2016; Yamashita et al., 2014). In addition, using powerful genome editing tools, such as clustered regulatory interspaced short palindromic repeat (CRISPR)-Cas9 system (Cong et al., 2013), mutations can be corrected in ACH stem cells *in vitro*. After screening and safety assessment in animal models *in vivo*, stem cell transplantation therapy may provide an effective and novel treatment (Qin and Gao, 2016).

In this study, we isolated and cultured skin, adipose and urine-derived somatic cells from ACH patients of Gly380Arg mutation and further generated non-integrated induced pluripotent stem cell (iPSC) lines by Sendai virus. Via powerful gene-editing tool - CRISPR-Cas9, we successfully obtained 2 completely corrected knock-in cell lines. Through cell immunofluorescence, karyotyping and sequencing analysis, these corrected iPSCs not only express pluripotent markers, maintained normal chromosomal number and structure, but also no off-target indels were identified. This is the first time to obtain ACH iPSCs using the most readily available biopsy sample - urine, the first to obtain non-integrated ACH iPSCs, and the first to perform gene correction in ACH research. Our study may provide important theoretical and experimental basis for the next step of stem cell research and treatment of ACH in the future.

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 children. Liposuction surgery was performed on adult man. All human subject protocols were reviewed and approved by the Ethical Review Board of the Ren Ji Hospital (Shanghai). All subjects gave signed informed consent.

isolation and culture of skin fibroblasts (SFs)

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

Culture and expansion of urine-derived cells (UCs)

The culture of UCs followed the method established by Pei laboratory (Zhou et al., 2012). Briefly, urine samples were collected into 50-mL tube and were centrifuged at 400 g for 10 minutes. The supernatants were aspirated carefully leaving only 1 mL in the tube. The pellets were resuspended by washing buffer (PBS containing ampothericin B (Selleck) and P/S) and were 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, CC-4127), ampothericin B and P/S. After 4 days, the medium was carefully changed to proliferation medium (REBM BulletKit (Lonza, CC-3190) to the REBMTM Renal Epithelial Cell Growth Basal Medium ((Lonza, CC-3191)).

Isolation and culture of adipose tissue-derived mesenchymal stem cells (AD-MSCs)

Adipose biopsy was obtained by liposuction surgery. Isolation and culture of AD-MSCs followed our previous reports (Qin et al., 2013; Qin et al, 2014; Qin et al., 2015b). Briefly, adipose tissues were subsequently digested with an equal volume of 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 the stromal vascular fraction. The pellets were suspended and cultured in fibroblast medium.

PCR and Sanger sequencing

Total genomic DNA was extracted from patient peripheral blood, skin, urine and adipose-derived cells 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* primer is 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 were 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 containing Sendai virus (SeV) vectors. 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, the colonies were picked for expansion and characterization.

Alkaline phosphatase (AP) staining

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

Design of single guide RNAs (sgRNAs) and vector construction

sgRNAs were designed to target point mutation site by using the Guide Design Resouces of Zhang lab (https://zlab.bio/guide-design-resources) and cloned into the pSpCas9(BB)-2A-RFP plasmids (Yang Hui Lab). In brief, the oligo sgRNA were annealed and ligated to the pSpCas9(BB)-2A-RFP plasmid which was digested with Bbs I (NEB).

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 irradiated mouse embryonic fibroblast (iMEFs) coated plates with ROCK inhibitor. 24-48 hours after electroporation, RFP positive cells were sorted and seeded on iMEFs coated plates by BD FACS AriaII. 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. The sequences of the primers for off-target sites were listed in Table 1.

Cell Immunofluorescence

Immunofluorescence was performed according to our previous report (Qin et al, 2015a). Firstly, iPSCs were fixed in 4% paraformaldehyde solution for 15 min at room temperature (RT). Next, they were permeabilized using 0.1% Triton X-100 in PBS for 20 min 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□. 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

Karyotyping was analyzed as we described previously (Qin et al, 2009). The iPSCs were cultured in E8 with 0.1 μg/mL colchicine (ThermoFisher) for 2 hours. After removing medium, they were incubated in 0.8% sodium citrate for 1 hours, 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 min. Finally, chromosome analysis was performed under the karyotyping image analysis system. More than 15 metaphase spreads of each sample were analyzed.

Results

Identification of ACH patients and generation of somatic cells from them

ACH patient donors included 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 they are all heterozygous mutations of a G-A transition at nucleotide 1138 of the cDNA, changing the codon for Gly380 (GGG) to Arg (AGG) in the transmembrane domain of *FGFR3* (Fig. 1A-C). In addition, their clinical phenotypes are consistent with the NIH-defined characteristics, such as short arms and legs, enlarged head and prominent forehead. Fibroblasts from 2 children gradually climbed out from skin and proliferated (Fig. 1D and E). AD-MSCs via lipoaspiration from adult man exhibited a typical fibroblast-like morphology (Fig. 1F). After 3-4 day of culture, adhered UCs from children formed colonies and showed epithelial cell morphology (Figure 1G and H). All somatic cells were enriched by serial plate passaging for next experiments.

Generation of iPSCs

3 weeks after transduction of SFs and UCs via SeV, lots of ES cell-like clones formed (Fig. 2A and C). Each single colony was pick up and expanded and a total of 70 iPSC lines were established (Fig. 2 B and D). iPSCs derived from girl's SFs and boy' UCs were used for pluripotency identification and characterization. The iPSCs not only expressed pluripotent marker - NANOG, OCT4, SOX2, SSEA-4 and TRA1-60 (but not express SSEA-1, Fig. 2G), but also showed AP positive (Fig. 2H and I). Karyotyping analysis indicated normal chromosomal number and structure (Fig. 2J and H). However, it is surprised that iPSCs can't be generated from AD-MSCs. Although small and unhealthy colonies can form (Fig. E), subsequently they gradually became apoptotic and died (Fig. E).

Gene correction of ACH iPSCs by CRISPR-Cas9

Around the point mutation site, 64 bp DNA sequences were entered into Guide Design Resouces and sgRNAs were generated. We selected the highest scored sg2RNA for use (Fig. 3A). To correct the point mutation in iPSCs from the ACH patient, 131 nucleotides acted as the homology arm ssODNs donor centered on point mutation site (Fig. 3A). FACS detection of transfected iPSCs by constructed CRISPR-sgRNAs and ssODNs showed the RFP positive cells were 3.6% (Fig. 3B). By sequencing analysis of 32 expanded single RFP positive colonies, we found 2 completely corrected knock-in cell lines (Fig. 3C and D), and the efficiency was 7.7% (Table 2). In addition, we also found two cell lines, that is, the knock-in (KI) that was correctly corrected, and the knock-out (KO) also occurred. In short, the efficiency of generating homology directed repair (HDR) is 12% (Table 2). We analyzed two precisely corrected iPSC lines and found they can continue to express pluripotent markers, NANOG, OCT4, SOX2, SSEA-4 and TRA1-60 (but not express SSEA-1, Fig. 4A), and indicate AP positive (Fig. 4B). The karyotyping analysis verified they maintained normal chromosomal number and structure (Fig. 4C). At last sequencing analysis of potential offtarget sites given by Guide Design Resouces showed that no off-target indels were identified.

Discussion

Whenever about 25,000 children are born, one of them may be an ACH patient (Horton et al., 2007). For heterozygous 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 (Horton et al., 2007). Moreover, the social concern of this disease is not high. With the rapid development of stem cell biology and gene-editing technology, the research of ACH is not only important, but also provides ideas and experimental basis for the investigation of other MGDs (Qin and Gao, 2016).

In this study, we first acquired three different tissue samples to culture somatic cells, including the most commonly used skin, the most easily available urine and large amount of acquired adipose tissue that can produce multipotent MSCs with chondrocyte differentiation ability. We found that, like skin cells, ACH patient urine-derived cells were also very efficiently reprogrammed into iPSCs, which may provide a new donor cells for stem cell research of ACH. Our study is the first time to obtain ACH iPSCs from urine and the first to obtain non-integrated ACH iPSCs. However, to our surprise, ACH patient-derived AD-MSCs cannot be reprogrammed into iPSCs. In fact, previously reported studies (Sugii et al., 2010; Sun et al., 2009) and our unpublished results found that healthy human AD-MSCs were reprogrammed to iPSCs more efficiently than skin cells. Given ACH is a chondrocyte generation disorder caused by *FGFR3* mutation, our results suggested that perhaps the point mutation affected the reprogramming ability of AD-MSCs.

By novel and powerful gene-editing tool - CRISPR-Cas9, we successfully obtained 2 completely corrected knock-in cell lines from urine-derived iPSCs, and the rate of HDR without KO was 7.7%, showing a optimistic efficiency comparing the studies already reported (Lin et al., 2014; Yang et al., 2016). These corrected iPSCs still displayed pluripotency and maintained a normal karyotype. Sequencing analysis of potential offtarget sites suggested no off-target indels were identified. Our study is the first to perform gene correction in ACH research.

In summary, we generated non-integrated iPSC lines from ACH patient skin and urine and successfully corrected these mutated stem cells. Our study will provide important theoretical and experimental basis for the next step of stem cell research and treatment of ACH in the future. 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 them will be pursued through further advances in basic research (Fig. 5).

Acknowledgement

This work was funded by grant from the National Natural Science Foundation of China (General Program 31570992 to Y.Q.). I would like to thank Dr. Wei-Qiang Gao for his helpful discussion in conceiving the project, Dr. Wenyuan Wang for providing the experimental platform, Dr. Hui Yang for providing pSpCas9(BB)-2A-RFP plasmid, Chikai Zhou for his helpful discussion in designing sgRNA, Dr. Sushan Luo and Fang Luo for skin biopsy and culture of skin cells. Mingfeng Guan and Yundong Li for their help of amplification of plasmid.

Author Disclosure Statement

There is no potential conflict of interest.

Figure legend

FIG. 1. Identification of ACH patients and generation of somatic cells from them. **(A-C)** Sequence of 3 ACH patient donors are all heterozygous mutation of a G-A transition at nucleotide 1138 of the cDNA. **(D and E)** SFs from girl and boy. **(F)** AD-MSCs from adult man exhibited a typical fibroblast-like morphology. **(G and H)** Adhered UC colonies from girl and boy showed epithelial cell morphology.

FIG. 2. Generation of non-integrated iPSCs and characterization of them. **(A)** 3 weeks after transfection ES cell-like colonies from girl SFs (GF). **(B)** Expanded iPSCs from GF. **(C)** 3 weeks after transfection ES cell-like colonies from boy UCs (BU). **(D)** Expanded iPSCs from BU. **(E)** 3 weeks after transfection ES

cell-like from AD-MSCs, they are small and unhealthy. **(F)** Colonies from AD-MSCs gradually became apoptotic and died. **(G and H)** iPSCs from GF and BU expressed pluripotent marker - NANOG, OCT4, SOX2, SSEA4 and TRA1-60, but not express SSEA-1. **(I)** iPSCs from GF and BU showed AP positive. **(J and H)** iPSCs from GF and BU indicated normal chromosomal number and structure.

FIG. 3. Gene correction of ACH iPSCs by CRISPR-Cas9. **(A)** Designed sgRNA and ssODNs around the point mutation site. **(B)** FACS detection showed the RFP positive cells were 3.6%. **(C and D)** Restored normal DNA sequence of 2 completely corrected knock-in cell lines.

FIG. 4. Characterization of corrected iPSCs. **(A)** Corrected iPSCs expressed NANOG, OCT4, SOX2, SSEA4 and TRA1-60, but not express SSEA-1. **(B)** Corrected iPSCs indicated AP positive. **(C)** Corrected iPSCs showed normal chromosomal number and structure.

FIG. 5. Diagrammatic strategy of ACH patient-derived stem cell research. Somatic cells can be 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, and undergo transplantation into ACH mouse models to assess their relative safety and therapeutic effects.

References

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819-823.

Hecht, J.T., Horton, W.A., Butler, I.J., Goldie, W.D., Miner, M.E., Shannon, R., Pauli, R.M. (1986). Foramen magnum stenosis in homozygous achondroplasia. Eur. J. Pediatr. 145, 545-547.

Horton, W.A., Hall, J.G., Hecht, J.T. (2007). Achondroplasia. Lancet 370, 162-172.

Lin, S., Staahl, B.T., Alla, R.K., Doudna. J.A. (2014). Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Elife 15, e04766.

Pauli, R.M., Conroy, M.M., Langer, L.O., Jr., McLone, D.G., Naidich, T., Franciosi, R., Ratner, I.M., Copps, S.C. (1983). Homozygous achondroplasia with survival beyond infancy. Am. J. Med. Genet. 16, 459-473.

Qin, Y. and Gao, W.Q. (2016). Concise Review: Patient-Derived Stem Cell Research for Monogenic Disorders. Stem Cells 34, 44-54.

Qin, Y., Ji, H., Wu, Y., Liu, H. (2019). Chromosomal instability of murine adipose tissue-derived mesenchymal stem cells in long-term culture and development of cloned embryos. Cloning Stem Cells

11, 445-452.

Qin, Y., Lin, J., Zhou, C., Yin, Q., Xie, Z., Zhang, X., Liu, X.Y., Gao, W., and Li, J. (2013). Mice cloned from white adipose tissue-derived cells. J. Mol. Cell. Biol. 5, 348–350.

Qin, Y., Qin, J., Zhou, C., Li, J., and Gao, W.Q. (2015a). Generation of embryonic stem cells from mouse adipose-tissue derived cells via somatic cell nuclear transfer. Cell Cycle 14, 1282-1290.

Qin, Y., Zhou, C., Wang, N., Yang, H., Gao, W.Q. (2015b). Conversion of adipose tissue-derived mesenchymal stem cells to neural stem cell-like cells by a single transcription factor, Sox2. Cell Reprogram. 17, 221-226.

Qin, Y., Zhou, P., Zhou, C., Li, J., and Gao, W.Q. (2014). The adipose-derived lineage-negative cells are enriched mesenchymal stem cells and promote limb ischemia recovery in mice. Stem Cells Dev. 23, 363-371.

Shiang, R., Thompson, L.M., Zhu, Y.Z., Church, D.M., Fielder, T.J., Bocian, M., Winokur, S.T., Wasmuth, J.J. (1994). Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. Cell 29, 335-342.

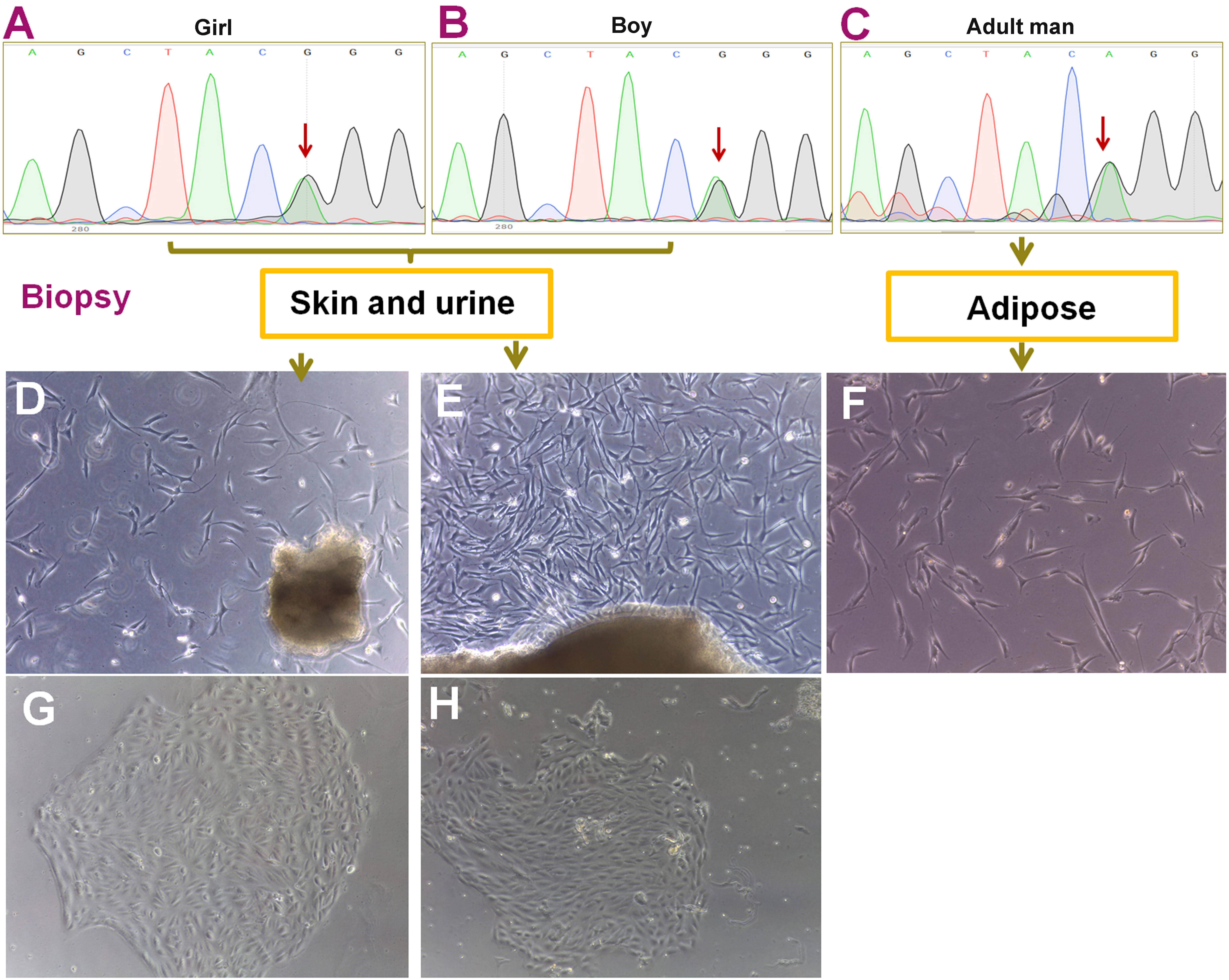
Superti-Furga, A., Eich, G., Bucher, H.U., Wisser, J., Giedion, A., Gitzelmann, R., Steinmann, B. (1995). A glycine 375-to-cysteine substitution in the transmembrane domain of the fibroblast growth factor receptor-3 in a newborn with achondroplasia. Eur. J. Pediatr. 154, 215-219.

Sun, N., Panetta, N.J., Gupta, D.M., Wilson, K.D., Lee, A., Jia, F., Hu, S., Cherry, A.M., Robbins, R.C., Longaker, M.T., Wu, J.C. (2009). Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. Proc. Natl. Acad. Sci. USA. 106, 15720-15725.

Sugii, S., Kida, Y., Kawamura, T., Suzuki, J., Vassena, R., Yin, Y.Q., Lutz, M.K., Berggren, W.T., Izpisúa Belmonte, J.C., Evans, R.M. (2010). Human and mouse adipose-derived cells support feeder-independent induction of pluripotent stem cells. Proc. Natl. Acad. Sci. USA. 107, 3558-3563.

Yamashita, A., Morioka, M., Kishi, H., Kimura, T., Yahara, Y., Okada, M., Fujita, K., Sawai, H., Ikegawa, S., Tsumaki, N. (2014). Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. Nature 513, 507-511

Yang, D., Scavuzzo, M.A., Chmielowiec, J., Sharp, R., Bajic, A., Borowiak, M. (2016). Enrichment of G2/M cell cycle phase in human pluripotent stem cells enhances HDR-mediated gene repair with customizable endonucleases. Sci. Rep. 18, 21264.


A
Girl
A G C T A C G G G
280
B
Boy
A G C T A C G G G
280
C
Adult man
A G C T A C A G G
Biopsy
Skin and urine
Adipose
D
E
F
G
H

A
B
C
D
E
F
G
Nanog
SSEA-1
Nanog/SSEA-1/DAPI
Oct4
SSEA-4
Oct4/SSEA-4/DAPI
GF
BU
Sox2
TRA-1-60
Sox2/TRA-1-60/DAPI
GF
BU
H
I
J
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18
K
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18

A
Human FGFR3 Seq
acgcccatgtctttgcagccgaggaggagctggtggaggctgacgaggcgggcagtgtgtatgcaggcatcctcagctacgggtgggcttcttcctgtt
11,100
tgcgggtacagaaacgtcggctcctcctcgaccacctccgactgctccgcccgtcacacatacgtccgtaggagtcgatgccccacccgaagaaggacaa
360 365 370 375 380 385
Ala Glu Glu Glu Leu Val Glu Ala Asp Glu Ala Gly Ser Val Tyr Ala Gly Ile Leu Ser Tyr Gly Val Gly Phe Phe Leu Phe
FGFR3
exon 10
Mutation
ACH FGFR3 Seq
sg2RNA
PAM
acgcccatgtctttgcagccgaggaggagctggtggaggctgacgaggcgggcagtgtgtatgcaggcatcctcagctacaggtgggcttcttcctgtt
11,100
tgcgggtacagaaacgtcggctcctcctcgaccacctccgactgctccgcccgtcacacatacgtccgtaggagtcgatgtcccacccgaagaaggacaa
360 365 370 375 380 385
Ala Glu Glu Glu Leu Val Glu Ala Asp Glu Ala Gly Ser Val Tyr Ala Gly Ile Leu Ser Tyr Arg Val Gly Phe Phe Leu Phe
FGFR3
exon 10
Correction
Gly
B
Specimen_001-ET
SSC-A (x 1,000)
FSC-A (x 1,000)
P1
Specimen_001-ET
SSC-W (x 1,000)
FSC-W (x 1,000)
Specimen_001-ET
SSC-A (x 1,000)
PE-Texas Red-A
P3
C
G C T T A C G G G
D
G C C T A C G G G

input Seq	ggcagtgtgtatgcaggcatcctcagctacagggtgggcttcttcctgttcatcctggtggtgg
sg2RNA-F	caccgatgcaggcatcctcagctac
sg2RNA-R	aaacgtagctgaggatgcctgcatc

ssODNs (131nt)

cagccgaggaggagctggtggaggctgacgaggcgggcagtgtgtatgcaggcatcctcagctacgggtgggcttcttcctgttcatcctggtggtggcggctgtgacgctctgccgcctgcgcagcccc

Tube: ET

Population	#Events	%Parent	%Total
All Events	14,672	####	100.0
P1	3,596	24.5	24.5
P2	3,070	85.4	20.9
P3	110	3.6	0.7

A
Nanog
SSEA-1
Nanog/SSEA-1/DAPI
Oct4
SSEA-4
Oct4/SSEA-4/DAPI
Sox2
TRA-1-60
Sox2/TRA-1-60/DAPI
B
C
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

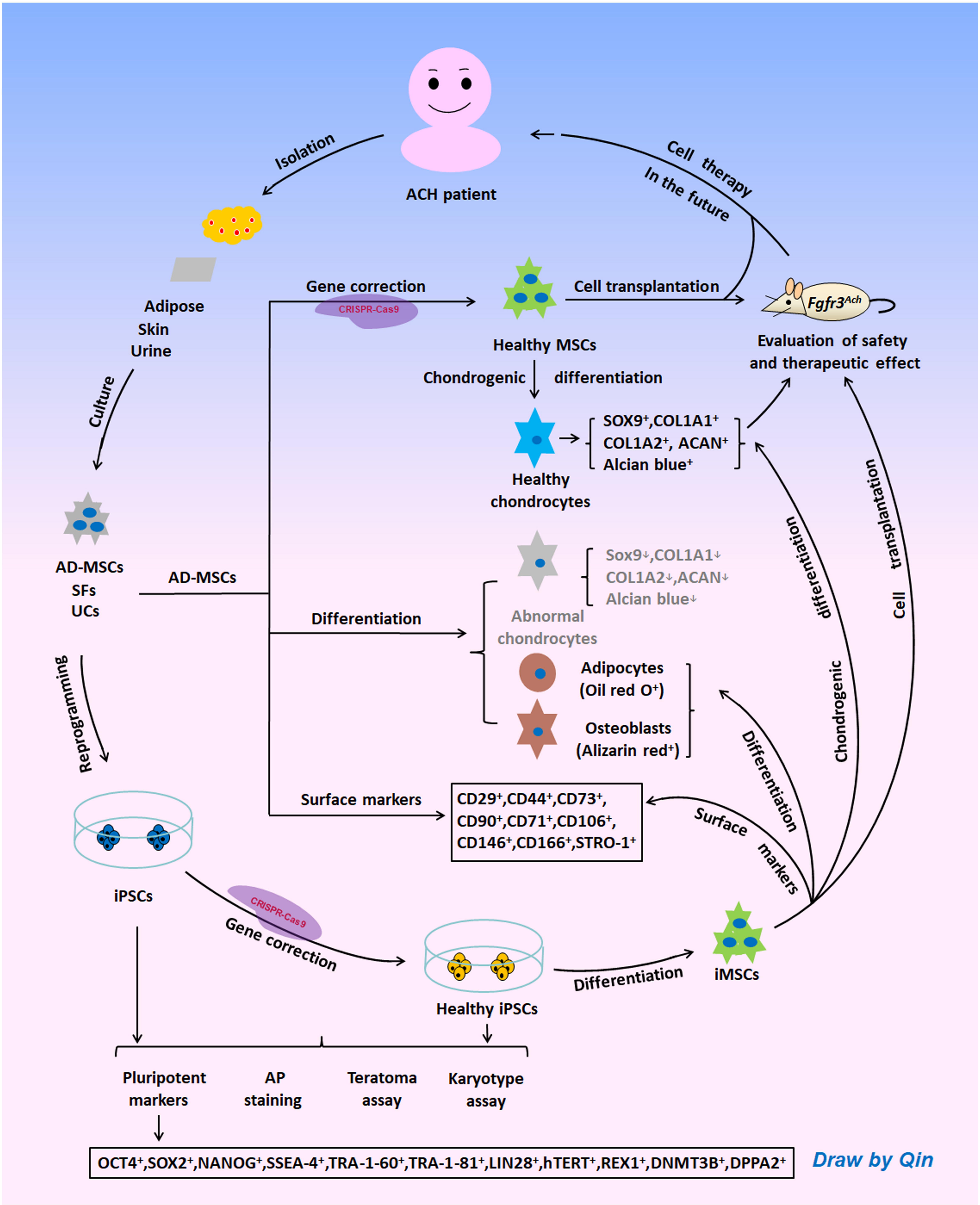

ACH patient
Isolation
Cell therapy
In the future
Adipose
Skin
Urine
Culture
Gene correction
CRISPR-Cas9
Healthy MSCs
Cell transplantation
Fgfr3^{Ach}
Evaluation of safety and therapeutic effect
Chondrogenic differentiation
SOX9$^+$,COL1A1$^+$
COL1A2$^+$, ACAN$^+$
Alcian blue$^+$
Healthy chondrocytes
AD-MSCs
SFs
UCs
AD-MSCs
Sox9↓,COL1A1↓
COL1A2↓,ACAN↓
Alcian blue↓
Abnormal chondrocytes
Differentiation
Adipocytes (Oil red O$^+$)
Osteoblasts (Alizarin red$^+$)
Chondrogenic differentiation
Cell transplantation
Reprogramming
Surface markers
CD29$^+$,CD44$^+$,CD73$^+$,
CD90$^+$,CD71$^+$,CD106$^+$,
CD146$^+$,CD166$^+$,STRO-1$^+$
Differentiation
Surface markers
iPSCs
CRISPR-Cas9
Gene correction
Healthy iPSCs
Differentiation
iMSCs
Pluripotent markers
AP staining
Teratoma assay
Karyotype assay
OCT4$^+$,SOX2$^+$,NANOG$^+$,SSEA-4$^+$,TRA-1-60$^+$,TRA-1-81$^+$,LIN28$^+$,hTERT$^+$,REX1$^+$,DNMT3B$^+$,DPPA2$^+$
Draw by Qin

TABLE 1. THE SEQUENCES OF THE PRIMERS FOR OFF-TARGET SITES

Gene name	Forward primer	Reverse primer
WNT11	AAGGAATTGGGGAAGCAGGG	GAGGATGAGGATGGTGCGAG
PRAG1	GCAGCTGCCCCGGCTCATCATCAGCAACT	CCGCTTCATGTCGATCCAGTTGTGCAGCG
GTF2E2	AGCTGAAGGGCTTCATCGTC	GAACTGCTCCTCTCCTCAGC
FLOT1	AGCCCATTCGTCTAAACTGGG	TTCCAGTTGCTTCTGTTTTATTAGT
CFAP45	TCCCTACCATTCTGCGAGGA	ATCCAAGCCCAAAGCAGTGA
SPTB	AAGTGATCCAAGGCCAGACG	GTGATGAGCACACCTCCCAA
FAM83E	TGGAGGCTCTGTTGAGCAAG	GGTTGTAGGCACCTGAGTCC
HGS	AGACACCTTCACTTGGGTGC	CAGTCAGGACCACACGCTTC
DSP	TGCTCCCAGGTTAGGCAAAG	GATTCCCCAAGCCACACTGA
TNNC1	CAGAGGCTTGGTCCCTCTTG	AGAGGCCAGGGTAGGTACTG
UBE2J1	CAATCATTCTTGGTAGTGTTGCCCTTAGTC	GTGAGACAAGGAACTTTGACTTCTAGTCCC
TYK2	GCATACCCCAACCCTGTCCAATAAATGTGG	GATGGGGCCCAGCTGCTGCTTGCACACC
ROBO4	GCCCAGTAGACCTGTCTGCCTCCTTTTAG	CACCTGTGTTTTAGGATGGCATCCTCACT
MYO15A	AGATGGGCAGCCATGGGAAGCTGTGAGTT	GCCTGAGACACCTCCAGGGCAGGAATGTG
HHLA1	CTGAAGGTGCAGCTACCAGGGGAACTGC	ACCCATACCGGCTCATACAGATCTGTTAT
TP53I3	GTATGACCCACCTCCAGGAGCCAGCAAC	TTCTGCTGGGCGTCCGGTGTTAACATGTAC
KIAA0100	ATCCTGGGAGATATGGTTCAAGCTATTCCA	CTTGCTGTGCCTCAGCTGGCTGGTTCCAAT
FCHSD1	ACCCTGCAGAGCACACAGGTACAGCAACA	CTTGAGCTTTGTGCTTGTGACCATGCTCA

TABLE 2. EFFICIENCY OF GEN-EDITING IN ACH IPSCS VIA CRISPR-CAS9

Colonies	**KO (NHEJ)**	**KI (HDR)**		**Homozygous mutant**	**No editing**	**No Seq**
		KI	**KI with KO**			
e1, e2, e3, e6, e7, e8, e9, e10, **e11**, e12, e13, e14, e16, e17, e18, e19, e20, e21, e22, e23, e24, e26, e27, e28, **e29**, e30, e31, e32, e33, e34, e35, e36	e1, e10 e14, e19 e24, e27 e32, e35	**e11, e29**	e8, e17	e16, e31, e34	e3, e6, e7, e12, e13, e20, e21, e22, e23, e28, e30, e33, e36	e2, e9, e18, e26
Total 32	**8 (25%)**	**2 (7.7%)**	**2 (6.3%)**	**3 (9.4%)**	**13 (40.6%)**	**4**
		4 (12.5%)				