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

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Abstract

Achondroplasia (ACH) is the most common genetic form of dwarfism and belong to dominant monogenetic disorder (MGD) caused by a mutation in the transmembrane region of fibroblast growth factor receptor 3 (*FGFR3*). 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 first acquired three different tissue samples from 3 ACH patients 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 mesenchymal stem cells (AD-MSCs) with chondrocyte differentiation ability. We found that, like skin cells, urine cells were also very efficiently reprogrammed into iPSCs, which may provide a new donor cells for stem cell research of ACH. However, AD-MSCs cannot be reprogrammed into iPSCs. 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 powerful gene-editing tool - CRISPR-Cas9, we successfully obtained 2 completely corrected knock-in cell lines from urine-derived iPSCs. 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 time to obtain ACH iPSCs from urine, the first to obtain non-integrated ACH iPSCs, and the first to perform gene correction in ACH research.

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. However, to our surprise, ACH patient adipose-derived mesenchymal stem cells (AD-MSCs) cannot be reprogrammed into iPSCs. 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. 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).

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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.

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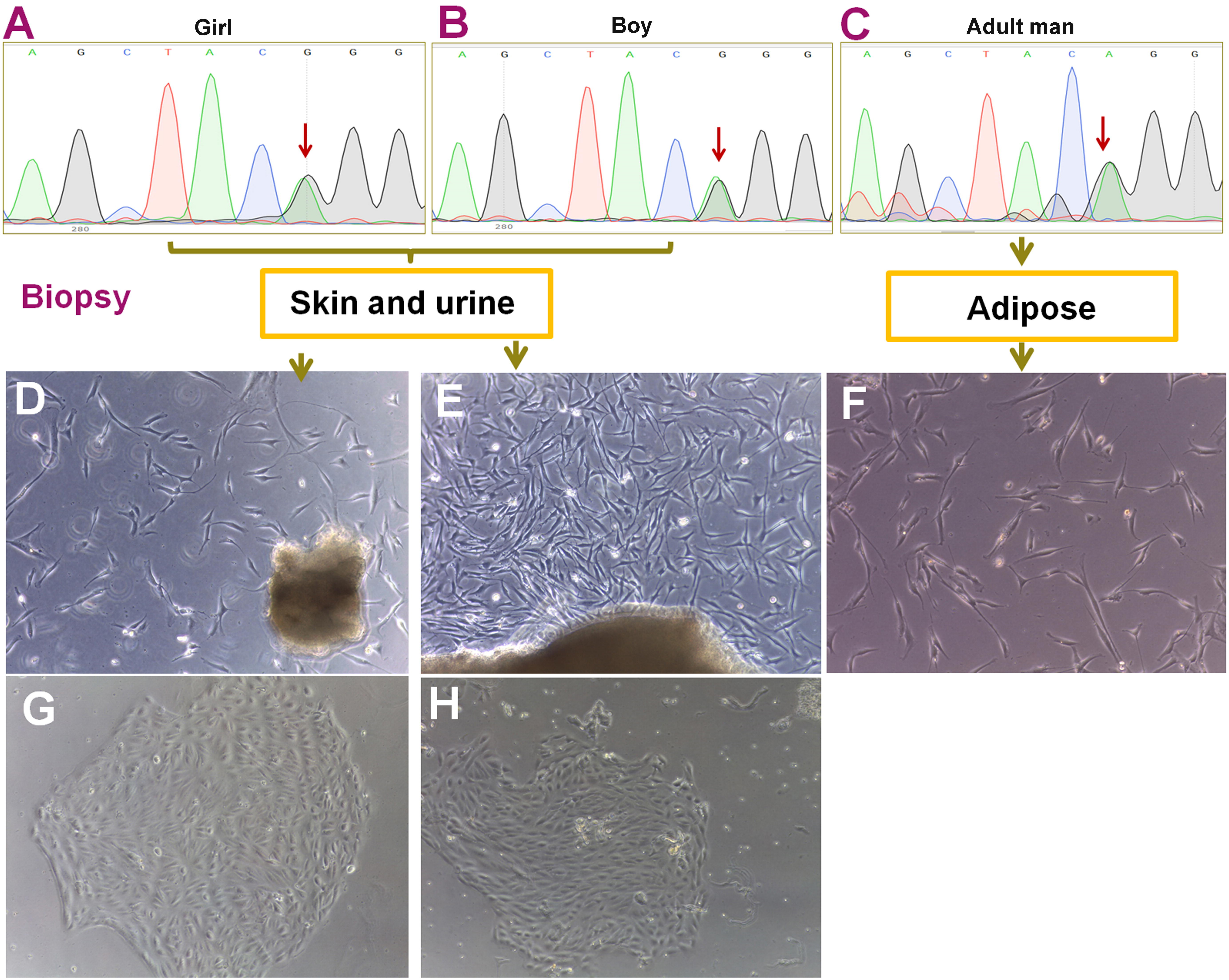
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Biopsy
Skin and urine
Adipose
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SSEA-1
Nanog/SSEA-1/DAPI
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Sox2/TRA-1-60/DAPI
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Ala Glu Glu Glu 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
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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)	

cagccgaggaggagctggtggaggctgacgaggcgggcagtgtgtatgcaggcatcctcagctacgggtgggcttcttcctgttcatc
ctggtggtggcggctgtgacgctctgccgcctgcgcagcccc

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
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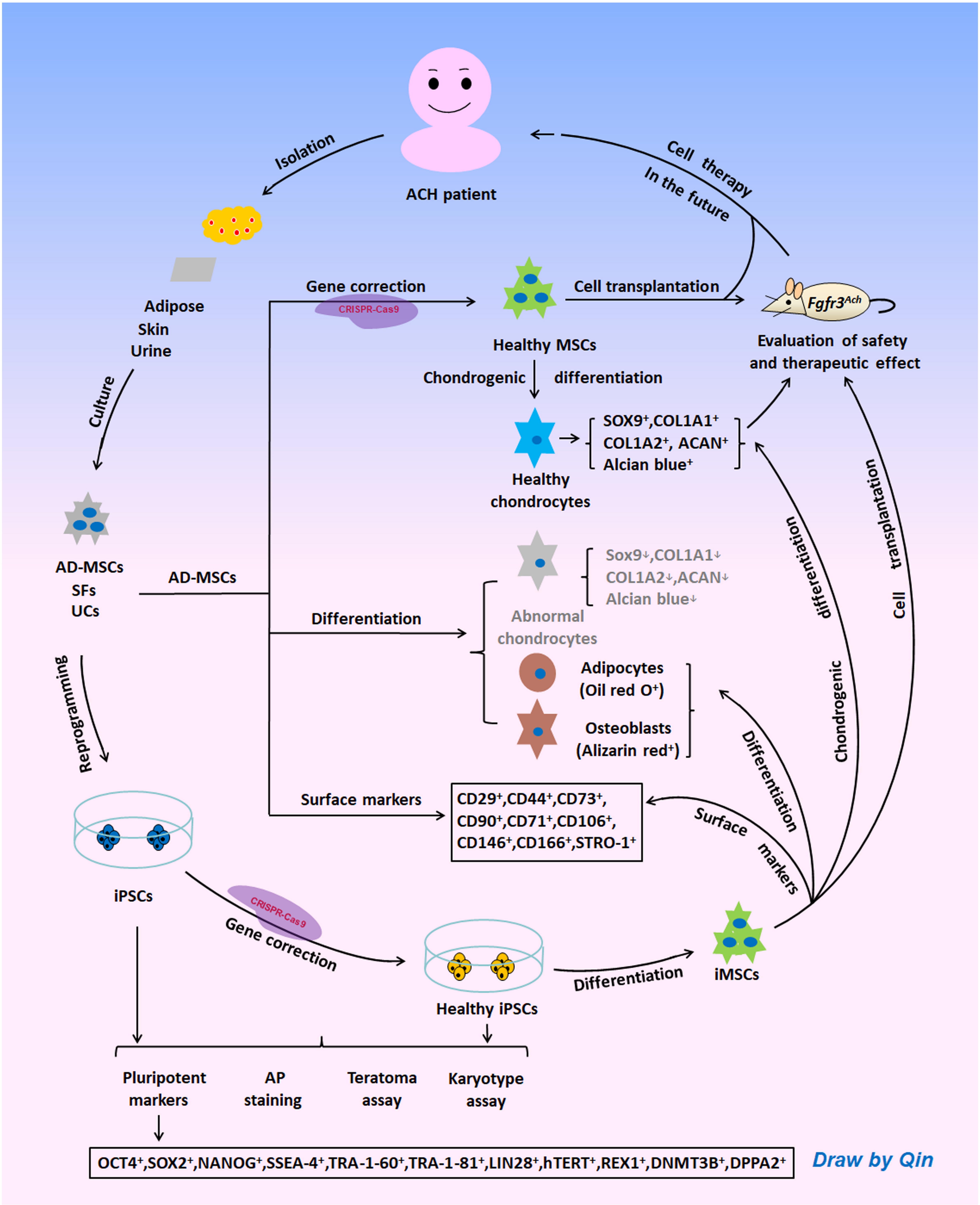
ACH patient
Isolation
Adipose
Skin
Urine
Culture
AD-MSCs
SFs
UCs
Reprogramming
iPSCs
AD-MSCs
Gene correction
CRISPR-Cas9
Healthy MSCs
Cell transplantation
Fgfr3^{Ach}
Evaluation of safety and therapeutic effect
Cell therapy
In the future
Chondrogenic differentiation
Healthy chondrocytes
SOX9$^+$,COL1A1$^+$
COL1A2$^+$, ACAN$^+$
Alcian blue$^+$
Differentiation
Abnormal chondrocytes
Sox9$\downarrow$,COL1A1$\downarrow$
COL1A2$\downarrow$,ACAN$\downarrow$
Alcian blue$\downarrow$
Adipocytes (Oil red O$^+$)
Osteoblasts (Alizarin red$^+$)
Surface markers
CD29$^+$,CD44$^+$,CD73$^+$,
CD90$^+$,CD71$^+$,CD106$^+$,
CD146$^+$,CD166$^+$,STRO-1$^+$
Chondrogenic differentiation
Cell transplantation
Differentiation
Surface markers
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$^+$
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