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Generation of human induced pluripotent stem cells from individuals with a homozygous CCR5 32 mutation

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Abstract

Chemokine receptor 5 (CCR5) is the primary coreceptor for HIV entry into macrophages. Individuals with a homozygous deletion of 32 bp in the *CCR5* gene (CCR5 32) are highly resistant to HIV infection (Samson et al., 1996). Allogeneic stem cell transplantation from a healthy donor with the homozygous CCR5 32 variant to an HIV positive individual has demonstrated efficient long-term control of HIV. We identified three individuals with this homozygous CCR5 32 variant, and successfully generated induced pluripotent stem cell (iPSC) lines from their dermal fibroblasts. The iPSCs lines carrying homozygous CCR5 32 variant displayed phenotypically normal and the potential to differentiation toward the three germ layers.

Resource table.

Unique stem cell lines identifier	NIHTVBi001-A NIHTVBi002-A NIHTVBi003-A
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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2019.101481>.

Alternative names of stem cell lines	iPSCp117 iPSCp118 iPSCp120
Institution	National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, Maryland, USA
Contact information of distributor	Manfred Boehm; boehmm@nhlbi.nih.gov
Type of cell lines	iPSC
Origin	Human
Cell Source	Dermal fibroblasts
Clonality	Clonal cell lines
Method of reprogramming	Lentiviral vectors
Multiline rationale	Lines derived from the three individuals
Gene modification	None
Type of modification	N/A
Associated disease	None
Gene/locus	CCR5, 3p21.31
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	December 2013
Cell line repository/bank	N/A
Ethical approval	National Institutes of Health Ethics Committee (Approval Number: 10-H-0126)

Resource utility

Human induced pluripotent stem cells (hiPSCs) containing CCR5 $\Delta 32$ provide an important model to better understand the mechanisms regulating the functions of CCR5 in immune cells and HIV pathophysiology. Meanwhile, these iPSCs could offer a novel strategy for developing stem cell therapy to treat individuals with HIV/AIDS.

Resource details

CCR5 is a receptor for β -chemokines, including macrophage inflammation proteins 1 α and 1 β and RANTES. The expression and regulation of CCR5 in human immune cells are implicated not only in inflammatory diseases but are also involved in viral infections such as HIV (Samson et al., 1996; Berger et al., 1999). This observation makes CCR5 an attractive target for developing treatment for individuals with HIV/AIDS. Several research groups have attempted to disrupt the *CCR5* gene in CD34⁺ hematopoietic stem/progenitor cells, CD4⁺ T cells, and hiPSCs by using gene editing. However, the effects of integration mutagenesis could potentially cause other complications, such as safety issues relating to long-term treatment (Li et al., 2013). The naturally occurring CCR5 $\Delta 32$ variant is barely rare; it has been observed in less than 1% of most European-derived populations and is less common in other racial groups. Other than HIV-resistance, there are no known clinical effects of the homozygous CCR5 $\Delta 32$ genotype (Martinson et al., 1997).

We identified three individuals homozygous for the CCR5 32 variant by exome sequencing and enrolled them into one of our NHLBI clinical protocols (10-H-0126) for further investigations. Information regarding clinical onset were obtained using the standard clinical interview (Table 1). Skin punch biopsy samples from these three individuals were collected at the NIH Clinical Center. Using a Cre/loxP excisable lentiviral vector delivery system expressing four transcription factors (*OCT4*, *SOX2*, *KLF4*, and *C-MYC*), we successfully generated hiPSC lines from skin fibroblasts derived from three individuals with the naturally occurring homozygous CCR5 32 mutation (hiPSC-CCR5 32) and from healthy volunteers (hiPSC-Con) who did not have that variant. The reprogramming efficiency of these skin fibroblasts was the same for both groups (Data not shown). The hiPSC-CCR5 32 lines maintained typical morphologies and expressed the typical pluripotency markers OCT4, NANOG, TRA-1-60, SSEA4, and SOX2, as shown by immunocytochemistry (Fig. 1A) and/or real-time (RT)-qPCR (Fig. 1C). Genotyping of the generated hiPSC-CCR5 32 lines showed a 32-bp deletion in *CCR5* in all clones that were the same as their parental fibroblasts (Fig. 1B). All three cell lines demonstrated chromosomal stability and a normal karyotype with G-banding (Fig. 1D).

To test the differentiation potential of the cell lines, we performed a monolayer differentiation assay to drive the cells toward the three germ layers *in vitro*. We determined the marker gene expression for the mesoderm (*RUNX1*), endoderm (*AFP*), and ectoderm (*WES*) with RT-qPCR, which showed comparable expression levels between the hiPSC-CCR5 32 and hiPSC-Con line (Fig. 1C). Short tandem repeat (STR) profiles indicated that all hiPSC-CCR5 32 lines matched with their parental fibroblasts completely in 15 amplified STR loci (see Supplementary File 1). All cultures were routinely tested for *Mycoplasma* contamination and were found to be *Mycoplasma* free as shown in Supplementary File 2. Notably, CCR5 was not expressed in iPSCs. hiPSC-CCR5 32 lines exhibited a similar pluripotent potential for self-renew and proliferation as hiPSC-Con line, suggesting that CCR5 is dispensable for reprogramming of fibroblasts and maintenance of stemness.

To the best of our knowledge, this is the first published study in which hiPSC lines were generated from individuals with the naturally occurring a homozygous CCR5 32 mutation (Table 2).

Materials and methods

Subjects and study approval

iPSCs were generated from fibroblasts derived from skin punch biopsy samples obtained from three individuals with the homozygous CCR5 32 variant and healthy volunteers. This study was approved by the NHLBI's institutional review board, and samples were collected after obtaining informed written consents.

Derivation of fibroblasts

Fibroblasts derived from individuals with the homozygous CCR5 32 variant and healthy volunteers were collected from explants of 4-mm skin punch biopsy specimens and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and

1% penicillin-streptomycin, as previously described (Jin et al., 2016). After 1–2 weeks, fibroblast outgrowths from the explants were passaged.

Generation and culture of human iPSCs from fibroblasts

Fibroblasts from individuals with the homozygous *CCR5* 32 variant were reprogrammed to generate iPSC lines by transduction with the Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Millipore). iPSC colonies were collected at 21 days post-transduction, and expanded in a typical hESC/iPSC culture condition (Jin et al., 2016).

Immunofluorescent staining

iPSCs were fixed with 4% paraformaldehyde and stained following the previous protocol (Jin et al., 2016). In brief, cells were incubated with primary antibodies against NANOG, OCT4, SSEA4, or TRA-1-60 (Table 3) at 4 °C overnight. Following washing with PBS, they were incubated with appropriate fluorophore-tagged secondary antibodies at room temperature for 1 h. After washing with PBS, nuclei were stained with DAPI. Images were captured using a fluorescence microscope (Zeiss).

Monolayer differentiation assay

To assess iPSCs' ability to differentiate *in vitro*, cell cultures were dissociated into small clumps with 0.5 μM EDTA and cultured on Matrigel Precoated Plates (Corning) with differentiation medium consisting of 90% KnockOut DMEM, 10% FBS, 2mM L-glutamine, 0.1 mM non-essential amino acids, and 0.1 mM 2-mercaptoethanol (Invitrogen). After seven days, cells were harvested for further analysis.

Gene expression analysis

The total RNA was isolated by using RNeasy Mini Kits (Qiagen). Endogenous mRNA expression levels of *NANOG*, *SOX2*, *AFP*, *NES*, and *RUNX1* were determined in iPSCs and in differentiating cells at day 7. For this, RT-qPCR was performed by using SYBR Green Premix on a Real-Time PCR Detection System (Bio-Rad). Assays were run in triplicate and the results were normalized to 18S ribosomal RNA expression. Primers used for RT-qPCR are shown in Table 3.

Karyotyping assay

The karyotype of the iPSCs was evaluated by the WiCell Research Institute using G-banding metaphase karyotype analysis.

DNA sequencing and STR

Genomic DNA was extracted by using DNeasy Blood & Tissue Kit (Qiagen). To amplify the corresponding deletion position in *CCR5*, PCR was performed with specific primers (Table 3). Following purification, the PCR products were sent to Eurofins Scientific for sequencing.

STR analysis was performed by WiCell Research Institute, which generated a STR profile *via* the Promega Powerplex® 16 System to verify STR polymorphisms for 15 loci plus amelogenin in genomic DNA extracted from iPSCs and their parental fibroblasts.

Mycoplasma detection

To validate the cultures of derived iPSCs were *Mycoplasma* free, media were collected after culturing for 48 h and analyzed by using the MycoAlert™ Mycoplasma Detection Kit (Lonza).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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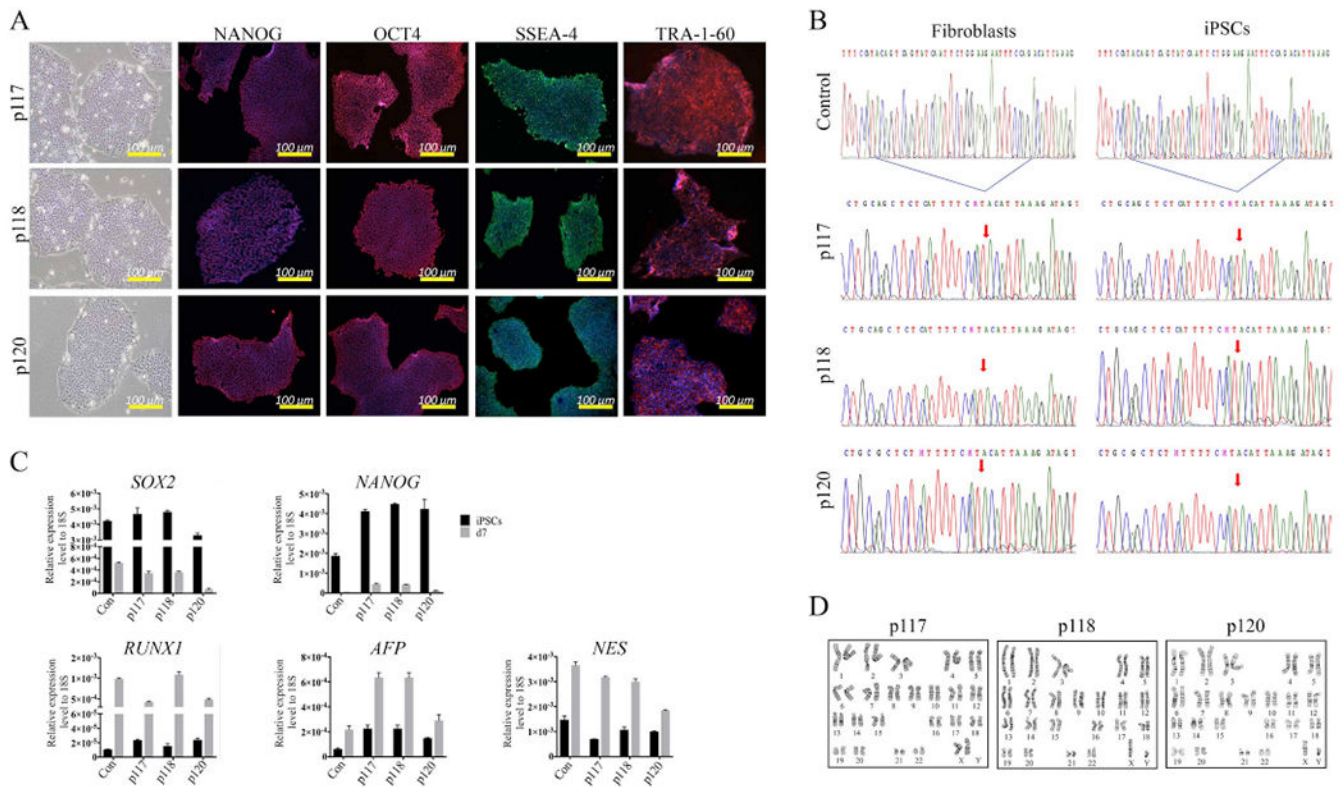


Fig. 1. Characterization of human iPSC lines derived from three individuals homozygous for the CCR5 32 variant. (A) iPSCs were cultured to passage 15 on a feeder-coated plate. Phase contrast images of iPSC lines derived from three individuals homozygous for the CCR5 32-bp deletion (hiPSC-CCR5 32; lines p117, p118 and p120) (column 1). Expression of pluripotent markers (NANOG, OCT4, SSEA4, and TRA-1-60) was analyzed by immunofluorescence; DAPI staining of cell nuclei in blue (columns 2-5) (all scale bars: 100 μ m). (B) PCR and DNA sequencing identified the 32-bp deletion in CCR5 in parental fibroblasts (top row) the iPSC lines (bottom row) from the three individuals (blue arrows), but not in those from a healthy volunteer (control). (C) Expression of pluripotent state genes (NANOG and SOX2) was confirmed in all hiPSC-CCR5 32 lines as assessed by RT-qPCR. All three hiPSC-CCR5 32 lines were able to differentiate into three germ layers using monolayer differentiation in vitro, as shown by gene expression of AFP, NESTIN and RUNX1. Data are represented as means \pm SEM relative to mRNA levels. (D) All three hiPSC-CCR5 32 lines had a normal karyotype by G-band analysis.

Table 1

Summary of three individuals with a homozygous CCR5 32 variant.

iPSC line names	Abbreviation in figures	Gender	Age (years)	Ethnicity	Genotype of locus	Disease
NIHTVB001-A	p117	F	60	European-American	CCR5, 3p21.31	None
NIHTVB002-A	p118	M	56	European-American	CCR5, 3p21.31	None
NIHTVB003-A	p120	M	69	European-American	CCR5, 3p21.31	None

Table 2

Characterization and validation.

Classification	Test	Result	Data
Morphology	Phase-contrast microscope	Normal	Fig. 1A
Phenotype	Qualitative analysis (immunofluorescence staining)	Expression of pluripotency markers: OCT4, NANOG, SSEA4 and TRA-1-60	Fig. 1A
Genotype	Quantitative analysis (RT-qPCR)	Expression of pluripotency markers: <i>SOX2</i> and <i>NANOG</i>	Fig. 1C
Identity	Karyotype (G-banding) and resolution	46,XX or 46,XY; resolution 450–500 bands	Fig. 1D
	Microsatellite PCR OR STR analysis	Not performed	N/A
	DNA sequencing	15 sites tested, 100% match	Supplementary file
Mutation analysis (IF APPLICABLE)	Southern blot OR WGS	Homozygous, <i>CCR5</i> 32-bp deletion	Fig. 1B
Microbiology and virology	<i>Mycoplasma</i> testing by luminescence	Not performed	N/A
Differentiation potential	Monolayer differentiation assay	Negative	Available from authors
Donor screening (OPTIONAL)	HIV1 + HIV2, hepatitis B virus, hepatitis C virus	Differentiating cells are expression of <i>RUNX1</i> , <i>AFP</i> , and <i>NE5</i> ; iPSCs were able to differentiate into three germ layers	Fig. 1C
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

Table 3:

Reagents.

Antibodies used for immunocytochemistry					
Antibody	Dilution	Company	Cat#	RRID	
Primary antibodies					
Rabbit anti-OCT4	1:100	Cell Signaling Technology	2750	AB_823583	
Mouse anti-NANOG	1:100	Cell Signaling Technology	4893	AB_10548762	
Mouse anti-SSEA4	1:100	MilliporeSigma	MAB4304	AB_177629	
Mouse anti-TRA-1-60	1:150	MilliporeSigma	MAB4360	AB_2119183	
Secondary antibodies					
Alexa Fluor 594 Donkey anti-rabbit	1:300	Life Technologies	A21207	AB_141637	
Alexa Fluor 594 Donkey anti-mouse	1:300	Life Technologies	A21203	AB_141633	
Alexa Fluor 488 Donkey anti-mouse	1:300	Life Technologies	A21202	AB_141607	
Alexa Fluor 555 Goat anti-mouse	1:300	Life Technologies	A21426	AB_2535847	
Primers used for RT-qPCR and PCR					
Target	Forward/reverse primer (5'-3')				
<i>NANOG</i>	AGG GAA ACA ACC CAC TTC T/CCT TCI GCG TCA CAC CAT T				
<i>SOX2</i>	CCC AGC AGA CTT CAC ATG T/CCT CCC ATT TCC CTC GTT TT				
<i>AFP</i>	AGC TTG GTG GAT GAA AC/CCC TCI TCA GCA AAG CAG AC				
<i>NESTIN</i>	GCG TTG GAA CAG AGG TTG GA/TGG GAG CAA AGA TCC AAG AC				
<i>RUNX1</i>	CTG CCC ATC GCT TTC AAG GT/GCC GAG TAG TTT TCA TTG CC				
<i>CCR5</i>	CTC CCA GGA ATC TTT ACC/TCA TTT CGA CAC CGA AGC AG				