Induced pluripotent stem cell line from a mouse model of human azoospermia with a frameshift mutation Tex11_1260Ins(TT)

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Abstract

Infertility is a common disease that impacts 15% of reproductive age couples worldwide, and genetic causes are implicated in about half of those cases. Non-obstructive azoospermia is a severe form of male infertility that features spermatogenic failure resulting in no sperm in the ejaculate and severely reduces the chance to have biological children. We created a Tex11_1260Ins(TT) (1260GATA → TTGGTA) mutant mouse that models the Tex11_1258(TT) mutation identified from a patient with nonobstructive azoospermia. The Tex11_1260Ins(TT) iPSC cells displayed characteristics of pluripotent-like morphology, expressed pluripotent protein markers, show normal karyotype, and can to differentiate into tissues of the three germ layers.

1. Resource table

| Unique stem cell line identifier | MMRi001-A-3 |
|----------------------------------|-------------|
| Alternative name(s) of stem cell line | Tex11_1260Ins(TT) |
| Institution | Magee-Womens Research Institute, University of Pittsburgh School of Medicine |
| Contact information of the reported cell line distributor | Kyle E. Orwig, orwigke@upmc.edu |
| Type of cell line | iPSC |
| Origin | Mouse |
| Additional origin info (applicable for human ESC or iPSC) | N/A |
| Cell Source | Dermal fibroblasts |
| Method of reprogramming | Sendai virus |
| Clonality | Clonal |

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Declaration of Competing Interest
The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Doungkamchan and Orwig has patent #PCT/US2018/043948 pending to University of Pittsburgh.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102728.
Evidence of the reprogramming transgene loss (including genomic copy if applicable)  
RT-PCR for Sendai virus

Cell culture system used  
Feeder-dependent on mouse embryonic fibroblasts (MEFs)

Type of Genetic Modification  
A frameshift mutation Tex11_1260Ins(TT) resulted from the change of GATG sequence to the TTGGTA sequence at the aspartic acid position 435 in exon 16

Associated disease  
Sterility/Infertility

Gene/locus  
Tex11 gene, Xq13.1

Method of modification/site-specific nuclease used  
No modification

Site-specific nuclease (SSN) delivery method  
N/A

All genetic material introduced into the cells  
N/A

Analysis of the nuclease-targeted allele status  
PCR amplification and Sanger sequencing for the targeted mutation Tex11_1260Ins(TT)

Method of the off-target nuclease activity surveillance  
N/A

Name of transgene  
N/A

Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)  
N/A

Inducible/constitutive system details  
N/A

Date archived/stock date  
November 2020

Cell line repository/bank  
Magee-Womens Research Institute

Ethical/GMO work approvals  
This study was approved by the ethical committee of the University of Pittsburgh (IACUC 17050289), Pittsburgh, PA, USA

Addgene/public access repository recombinant DNA sources’ disclaimers (if applicable)  
N/A

2. Resource utility

A *TEX11* _1258Ins(TT)_(1258GATA → TTGGTA; D435) mutation was identified in a patient with non-obstructive azoospermia (Yang et al., 2015). Tex11_1260Ins(TT) (1260GATA → TTGGTA; D421) male mice recapitulate the meiotic arrest phenotype observed in the azoospermic patient (Wang et al., 2021). Tex11_1260Ins(TT) mouse iPSCs and in vitro gametogenesis can be used to understand the infertility phenotype and develop targeted therapies.

3. Resource details

Azoospermia is defined as a complete absence of sperm in the ejaculate and affects 1% of men worldwide (Gudeloglu and Parekattil, 2013). Non-obstructive azoospermia (NOA) accounts for 80–85% of all azoospermic cases, of which, 75% are idiopathic (unexplained) and difficult to treat. The other 15–20% are obstructive azoospermia (OA, blockage in the excurrent duct system) that is easily treated by recovering sperm directly from the testes (Gudeloglu and Parekattil, 2013). Mutations in the Tex11 gene; an X-linked gene, were found at high prevalence in two independent studies (Yang et al., 2015; Yatsenko et al., 2015). Yang and colleagues identified a frameshift mutation (GATG → TTGGTA; *TEX11* _1258Ins(TT)_ at the aspartic acid position 435 in exon 16 of *TEX11* in a NOA patient who was diagnosed with meiotic arrest at pachytene (Yang et al., 2015). The
TEX11_1258Ins(TT) human mutation was modeled by mutating the homologous locus in Tex11_1260Ins(TT) mice that exhibit the same NOA phenotype with maturation arrest at the pachytene spermatocyte stage (Wang et al., 2021). We produced the same mouse model, confirmed the NOA phenotype and established the MWRIi001-A-3 iPSC line from skin fibroblasts.

Skin fibroblasts isolated from adult Tex11_1260Ins(TT) male mouse were used to generate MWRIi001-A-3 iPSCs using non-integrated Sendai virus (SeV) to deliver mRNAs of the Yamanaka reprogramming factors Oct4, Sox2, Klf4, and c-Myc in a feeder-dependent manner. After 1 week, iPSC colonies emerged and were manually picked to be reseeded on mouse embryonic fibroblast (MEF) feeder cells. After a few passages, the MWRIi001-A-3 iPSC line was established and maintained its morphology of round, dome-shaped colonies with smooth edges (Fig. 1A). PCR amplification and Sanger sequencing confirmed that the MWRIi001-A-3 iPSCs contained the Tex11_1260Ins(TT) genotype (Fig. 1B–C) and were male (Fig. 1D). After 16 passages, the MWRIi001-A-3 iPSC line was tested for absence of SeV vector by RT-PCR using specific primers (Fig. 1E). Furthermore, cells from MWRIi001-A-3 had a normal karyotype (40, XY) (Fig. 1F).

Immunofluorescence staining of putative MWRIi001-A-3 iPSCs revealed expression of pluripotency markers Alkaline Phosphatase (AP), SSEA1, OCT4, SOX2, DPPA2, NANOG (Fig. 1G–H). MWRIi001-A-3 iPSCs were also confirmed to be mycoplasma-free at passage 16 (Supplementary figure 1). MWRIi001-A-3 cells can be harvested and cryopreserved in liquid nitrogen for long-term storage.

At passage 8, the differentiation capacity of the MWRIi001-A-3 cells into three germ layers was determined by conventional teratoma assay. Histological evaluation revealed tissue differentiation to all three germ layers within the teratoma, including endoderm (gastrointestine, bone marrow), mesoderm (smooth muscle, bone) and ectoderm (neurorosette, skin) (Fig. 1I) (each indicated tissue was marked with the arrow).

Our results demonstrated that we have produced a stable iPSC line from an infertile mouse model carrying a frameshift mutation Tex11_1260Ins(TT) that was identified in an idiopathic NOA patient with the homologous mutation (TEX11_1258Ins(TT)). MWRIi001-A-3 cell line can be used with in vitro gametogenesis methods to understand the mechanisms of spermatogenic failure caused by this specific patient-derived mutation and to develop targeted treatments (e.g., CRISPR/Cas9 gene editing) to repair the mutant allele.

4. Materials and methods

4.1. Fibroblast isolation and reprogramming

Skin tissue pieces from an adult TEX11_1258Ins(TT) male mouse were used to derive fibroblasts. At passage 2, these fibroblasts were transduced with non-integrated CytoTune iPS 2.0 Sendai Reprograming Kit (ThermoFisher). Seven days post-induction with Sendai viruses, putative iPSCs (MWRIi001-A-3) were seeded on mitomycin-C treated mouse embryonic fibroblasts (MEF) in GlutaMAX DMEM/F12 (ThermoFisher), 20% Knockout Serum Replacement-Mutiple Species (ThermoFisher), 1% NEAA, 1%
2-Methylmethacathinone (ThermoFisher), and 1ug mouse Leukemia Inhibitory Factor (Invitrogen). iPSCs were cultured in 37 °C, 5% CO2, and passaged using TrypLE (ThermoFisher) as single cells every 3–5 days.

4.2. Sanger sequencing analysis
Genomic DNA of MWRIi001-A-3 iPSCs, Tex11_1260Ins(TT) cells and wild-type mouse iPSC cells were extracted using QIAamp Micro DNA kit (QIAGEN). DNA was mixed with Tag 2X Master Mix (BioLabs) and specific primers (Table 2) following these PCR cycle parameters: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, repeated for 35 cycles, followed by a final extension at 72 °C for 10 min. PCR products were analyzed by agarose gel electrophoresis, then were purified using the QIAquick PCR Purification Kit (QIAGEN) and sent to the University of Pittsburgh Genomics Research Core for Sanger sequencing.

4.3. Reverse transcription PCR analysis of SeV vectors
After passage 16, total RNA was extracted from MWRIi001-A-3 iPSCs using RNeasy MiniKit (QIAGEN). RNA from transduced cells at passage 3 was used as the positive control. RT-PCRs for the detection of Sendai transgenes were performed using the GoScript Reverse Transcriptase cDNA synthesis kit (Promega). SeV specific primers were used to assess the presence of remaining Sendai virus (SeV) vectors (Table 2).

4.4. Karyotype analysis
Metaphase chromosomes were prepared from MWRIi001-A-3 iPSCs at passage 4. Twenty metaphase spreads were analyzed and karyotyped by the Cytogenetics and Molecular Pathology Laboratory at Washington University, St. Louis.

4.5. Immunofluorescence staining for pluripotency markers
At passage 5, the pluripotent status of putative MWRIi001-A-3 iPSCs was evaluated by immunofluorescent staining for four six markers (AP, SSEA1, SOX2, OCT4, DPPA2, NANOG) according to instructions in the Fluorescent Mouse ES/iPS Cell Characterization Kit (Millipore) (Table 1).

4.6. Flow cytometry analysis for pluripotent markers
At passage 17, MWRIi001-A-3 iPSCs were harvested by TryLE and incubated for 45 min at 4 °C with conjugated surface marker anti-SSEA1 antibody (Table 2). For nuclear marker staining, cells were first fixed and permealized using CytoFix/CytoPerm (Fisher Scientific) before incubation with conjugated anti-SOX2 or anti-OCT4 antibody (Table 2). A published mouse iPSC line (ALSTEM) was used as the control. FCS Express 6 software was used for the flow cytometry analysis.

4.7. Teratoma assay
MWRIi001-A-3 iPSCs were harvested using TryPLE, and 1×10⁶ cells in 8 μl mPSC medium was injected into the interstitial space of 6-week-old NOD/SCID mouse testes. After 2–4
months, tumors were harvested, fixed in Bouins, histologically processed and stained with hematoxilin/eosin for teratoma analysis.

4.8. Short tandem repeat (STR) analysis

STR analysis was performed by the ATCC Cell Line Authentication Service.

4.9. Mycoplasma test

The LookOut Mycoplasma PCR Detection Kit (Sigma) was used to detect mycoplasma contamination in iPSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Characterization of the Tex11_1260Ins(TT) induced pluripotent stem cell (iPSC) line derived from an infertile male mouse model. (A) Phase contrast image showing normal morphology of Tex11_1260Ins(TT) iPSCs growing on a feeder-coated plate (scale bar = 50 μm). (B) Genotyping PCR products of Tex11_1260Ins(TT) iPSCs to confirm their origin from Tex11_1260Ins(TT) mouse fibroblasts. Wild-type control iPSCs (labeled as (+) WT) were used as a control. (C) Sanger sequencing of PCR products for wild type iPSCs, Tex11_1260Ins(TT) fibroblasts, and Tex11_1260Ins(TT) iPSCs to confirm their genotype as wild-type (GATG) or mutant (TTGGTA). (D) PCR results confirming the sex
of $\text{Tex11}_{1260}\text{Ins}(TT)$ iPSCs as male using $F_{\text{Sry}}$-$R_{\text{Sry}}$ primer pair specific for the Sry gene. Cells of male (M) origin or female (F) origin were used as controls. Fc-Rv primer pair was used to confirm presence of DNA in all cell samples. (E) RT-PCR result showing absence of sendai virus (SeV) in $\text{Tex11}_{1260}\text{Ins}(TT)$ iPSCs at passage 16. $\text{Tex11}_{1260}\text{Ins}(TT)$ iPSCs at passage 3 were used as positive control for SeV. GAPDH housekeeping gene was used as a control for RT-PCR analysis. (F) $\text{Tex11}_{1260}\text{Ins}(TT)$ iPSCs showing normal karyotype of 40 chromosomes (mouse). (G) Immunofluorescent staining of $\text{Tex11}_{1260}\text{Ins}(TT)$ iPSCs showing positive staining for pluripotent markers Alkaline phosphatase (AP), SSEA1, SOX2, OCT4, NANOG, DPPA2. (H) Flow cytometry analysis showing no difference in percentage of SSEA1+, SOX2+, or OCT4 + $\text{Tex11}_{1260}\text{Ins}(TT)$ iPSCs compared to control iPSCs. (I) Teratoma assay analysis confirming tri-lineage (endoderm, mesoderm, ectoderm) differentiation potential of transplanted $\text{Tex11}_{1260}\text{Ins}(TT)$ iPSCs.
| Classification (optional italicized) | Test | Result | Data |
|-------------------------------------|------|--------|------|
| Morphology                          | Photography | Typical round, dome-shaped colonies with clear edges | Fig. 1A |
| Pluripotency status evidence for the described cell line | Qualitative analysis (Immunocytochemistry) | Positive staining for pluripotency markers (Alkaline Phosphatase (AP), SSEA1, SOX2, OCT4, DAPP2, NANOG) | Fig. 1G |
| Karyotype                           | Quantitative analysis | Flow cytometry (SSEA1 98.2%, SOX2 73.1%, OCT4 79.1%) | Fig. 1H |
| Genotyping for the desired genomic alteration/allelic status of the gene of interest | Karyotype (G-banding) | 40XY, 300–850 band resolution | Fig. 1F |
| Verification of the absence of random plasmid integration events | Mutant-specific PCR and Sanger sequencing | The Tex11_1260Ins(TT) mutation in MWRIi001-A-3 iPSCs was confirmed by PCR amplification with mutant primers (Fmt-Rv), but not by the wild-type primers (Fwt-Rv). Positive control primers (Fc-Rv) detected PCR product in both mutant and wild-type samples. Negative control (H2O) showed no PCR amplification. | Fig. 1B |
| Parental and modified cell line genetic identity evidence | Transgene-specific PCR | Not performed | N/A |
| Parental and modified cell line genetic identity evidence | PCR/Southern | The MWRIi001-A-3 iPSC line was tested for absence of SeV vector by RT-PCR using specific primers | Fig. 1E |
| Parental and modified cell line genetic identity evidence | Microsatellite PCR (mPCR) STR analysis | Not performed | N/A |
| Mutagenesis / genetic modification outcome analysis | Sequencing (genomic DNA PCR and Sanger sequencing) | X-linked hemizygous Tex11_1260Ins(TT) mutation | Fig. 1C |
| Mutagenesis / genetic modification outcome analysis | PCR-based analyses | Tex11_1260Ins(TT) mutation | Fig. 1B |
| Off-target nuclease analysis | Immunohistochemistry | Not performed | N/A |
| Specific pathogen-free status | PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing | Not performed | N/A |
| Specific pathogen-free status | Mycoplasma | No mycoplasma contamination | Supplementary Fig. 1 |
| Multilineage differentiation potential | Teratoma formation | Endoderm: gastrointestinal, bone marrow | Fig. 1I |
| Multilineage differentiation potential | | Mesoderm: smooth muscle, bone | |
| | | Ectoderm: neurorosette, skin | |
| Donor screening | HIV 1 + 2 Hepatitis B, Hepatitis C | Not performed | N/A |
| Classification (optional italicized) | Genotype - additional histocompatibility info | Blood group genotyping | HLA tissue Typing |
|------------------------------------|---------------------------------------------|------------------------|-----------------|
| Data                               | Not performed                              | Not performed          | N/A             |
| Result                             | N/A                                         | N/A                    | N/A             |

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### Table 2

#### Antibodies and stains used for immunocytochemistry

| Antibody                  | Dilution | Company Cat # and RRID     |
|---------------------------|----------|---------------------------|
| Alkaline phosphatase      | 1:2:1    | Millipore (Cat# SCR077)   |
| Mouse anti-SSEA1          | 1:100    | RRID: AB_2904506          |
| Mouse anti-SOX2           | 1:100    |                           |
| Mouse anti-OCT4           | 1:100    |                           |
| Mouse anti-NANOG          | 1:100    |                           |
| Mouse anti-DPPA2          | 1:100    |                           |
| **Nuclear stain**         |          |                           |
| DAPI                      | 1:1000   | Millipore (Cat# SCR077)   |
|                           |          | RRID: AB_2904506          |

#### Site-specific nuclease

| Nuclease information | Delivery method | Selection/enrichment strategy |
|----------------------|-----------------|------------------------------|
| N/A                  | N/A             | N/A                          |

#### Primers and Oligonucleotides used in this study

| Genotyping (PCR)         | Target                          | Forward/Reverse primer (5′–3′) |
|--------------------------|---------------------------------|--------------------------------|
| **Tex11 common sequence**| Fc-Rv (625 bp)                  | F: TGAAGGTATCTCCACTAGCATGG     |
|                          |                                 | R: ACCTAAGTGCCACAGAAAAGAAC     |
| **Tex11 wild-type**      | Fwt-Rv (202 bp)                 | F: GGTCCAAAAATATGCTGATG        |
|                          |                                 | R: ACCTAAGTGCCACAGAAAAGAAC     |
| **Tex11_1260ins(TT) mutant** | Fmt-Rv (206 bp)             | F: GGTCCAAAAATATGCTTTGTA       |
|                          |                                 | R: ACCTAAGTGCCACAGAAAAGAAC     |
| **Male genotype (Sry-positive)** | FSry-RSry (387 bp)          | F: TGGTCTGGACCCAAAACGCTGTCACACA |
|                          |                                 | R: GAGTACAGGTGTCAGCTCT         |
| **Sendai virus checking (RT-PCR)** | SeV (181 bp)             | F: GGATCACTAGGTGATATCGAC       |
|                          |                                 | R: ACCAGACAAGAGTTTAAGAGATAGTATC|
| **House-keeping gene**   | GAPDH (75 bp)                   | F: GACCAGCTCAAGGCTGAGAAC       |
|                          |                                 | R: AGGGATCTCGCTCCGGAA          |