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Lab resource: Stem Cell Line

Generation of WAe001-A-58 human embryonic stem cell line with inducible expression of the SARS-CoV-2 nucleocapsid protein

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A B S T R A C T

Excessive prostaglandin E_2 (PGE_2) is the key pathological basis for COVID-19 and a Celebrex treatment of hospitalized COVID-19 patients with comorbidities led to 100% discharged rate and zero death (Hong et al. 2020). It is also suggested that SARS-CoV-2 infected multiple organs and the SARS-CoV nucleocapsid (N) protein transcriptionally drives the expression of the host *COX-2* gene. In order to test whether SARS-CoV-2 N protein activates *COX-2* transcription in multiple human relevant cell types, an expression inducible human embryonic stem cell line was generated *by piggyBac* transposon system. This cell line maintained its pluripotency, differentiation potentials, normal morphology and karyotype.

1. Resource table

Unique stem cell line identifier	WAe001-A-58
Alternative name(s) of stem cell line	H1-SARS-CoV-2-N-Teton
Institution	Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences
Contact information of distributor	Yin-xiong Li, li_yinxiong@gibh.ac.cn
Type of cell line	Embryonic stem cell
Origin	Human
Additional origin info	Age: blastocyst stage Sex: Male, 46, XY
Cell Source	N/A
Clonality	Clonal
Method of reprogramming	N/A
Genetic Modification	YES
Type of Modification	Transgene expression
Associated disease	Coronavirus disease 2019 (COVID-19)
Gene/locus	N/GU280_gp10
Method of modification	*piggyBac* puromycin
Name of transgene or resistance	
Inducible/constitutive system	*TET*
Date archived/stock date	December 17, 2020
Cell line repository/bank	N/A
Ethical approval	Cell line was used according to institutional ethical guidelines

2. Resource utility

SARS-CoV-2 could infect multiple human stem cell-derived cells and organoids *in vitro* (Yang et al., 2020). Therefore, this cell line provides an ideal platform to illustrate the pathological role of SARS-CoV-2 N protein in multiple organoids and cell types, as well as to screen inhibitors for COX-2 and prostaglandins production (Table 1).

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3. Resource details

SARS-CoV-2 causes severe inflammation and damage to the lungs as well as other organs resulting in high mortality of COVID-19 patients. The molecular mechanism behind this event remained to be elucidated. Our recent study found that PGE_2 concentration was significantly elevated in COVID-19 and COX-2 specific inhibitor promoted recovery of this disease (Hong et al., 2020). Previous studies shown that SARS-CoV N protein could directly bind to the promoter of *COX-2* gene and stimulate COX-2 expression in a dose-dependent manner (Yan et al., 2006). The SARS-CoV-2 N protein shares high similarity with SARS-CoV N protein in the amino acid sequence and enhance nuclear localization signals (NLS) that may contribute to virus pathogenicity were identified in this protein (Gussow et al., 2020). Therefore, we speculate that SARS-CoV-2 N protein holds the potential to activate *COX-2* expression and subsequently leads to excessive prostaglandin E_2 production. Moreover, SARS-CoV-2-N could also interact with the host translation machinery by binding the stress granule (SG) proteins G3BP1/2, host mRNA binding proteins LARP1 and protein kinases CK2, and mRNA decay factors UPF1 and MOV10 (Gordon et al., 2020), which may further regulate the host antivirus response and viral replication.

The cell line WAe001-A-58 was generated from the human embryonic stem cell line WA01 (H1), with the Tet-On gene expression system and *piggyBac* transposon system. This *piggyBac* transposon system contains two plasmids: pB-TRE-EF1α-rtTA-2A-puroR-2A-EGFP and pBase. The SARS-CoV-2 N protein coding sequence (NCBI gene ID: 43740575) was inserted into the plasmid pB-TRE-EF1α-rtTA-2A-puroR-2A-EGFP and under the control of tetracycline-responsive promoter element (TRE). The validated construct was designated as pB-TRE-N-EF1α-rtTA-2A-puroR-2A-EGFP (Fig. 1A). In this plasmid, the coding sequences of RtTA, puromycin resistance and EGFP, each separated by a 2A sequence, were constitutive expressed under the control of EF1α promoter. Only in the presence of doxycycline will RtTA undergo conformational change, bind to the TRE and activate SARS-CoV-2 N protein expression. The integration of Tet-On system to the host cells was mediated by *piggyBac* transposase that expressed by pBase. Gene transfer was achieved by electroporation of the two plasmids into H1 cells. Selected puromycin resistant cells were distributed into 96-well plates for single cell expansion. Only those single-cell derived clones with intense green fluorescence were expanded, and one of them, WAe001-A-58 was selected for further characterization, as SARS-CoV-2 N protein is inducible in a doxycycline dose-dependent manner (Fig. 1D).

WAe001-A-58 cells showed a typical human embryonic stem cell morphology (Fig. 1.B), normal karyotype (46, XY) (Fig. 1.G), and were free of mycoplasma (Fig. 1 H). Immunofluorescence staining analyses showed that pluripotent markers OCT4 and NANOG were normally expressed in WAe001-A-58 cells (Fig. 1.C). Flow cytometric analysis measured the expression level of stem cell surface markers SSEA4 and TRA-1-60, which were 98.7% and 96%, respectively (Fig. 1.E). Furthermore, the cell line retained its differentiation potential *in vitro* as determined by the mRNA expression of the three germlines layer markers (*SOX1, PAX6, SOX17, CXCR4, MSX1*, and *TBX1*) (Fig. 1F). Short Tandem Repeat (STR) analyses confirmed that the WAe001-A-58 cells were derived from the H1 cell line.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel B
Phenotype	Immunofluorescence	OCT4 and NANOG positive	Fig. 1 panel C
	Flow cytometry	SSEA-4: 98.5% TRA-1-60: 96%	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	46 XY, Resolution 450–500	Fig. 1 panel G
Identity	Microsatellite PCR (mPCR)	Not done	Not done
	STR analysis	20 alleles were tested and 100% matched	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Not done	Not done
	Southern Blot OR WGS	Not done	Not done
Microbiology and virology	Mycoplasma	Negative	Fig. 1 panel H
Differentiation potential	Embryoid body formation	Express: SOX1, PAX6, SOX17, CXCR4, MSX1 and TBX1	Fig. 1 panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not done	Not done
Genotype additional info (OPTIONAL)	Blood group genotyping	Not done	Not done
	HLA tissue typing	No done	Not done

4. Materials and methods

4.1. Cell culture

WA01 (H1) and WAe001-A-58 cells were cultured on 1% Matrigel-coated plates in mTeSR1 medium (Stem cell) and maintained at 37 °C in humidified air with 5% CO_2. Cells were passaged with Accutase (Sigma) when 80% confluence was reached.

4.2. Plasmid construction and generation of WAe001-A-58

The pB-TRE-EF1α-rtTA-2A-puroR-2A-EGFP and pBase plasmids were kept in our lab. The SARS-CoV-2 N protein encoding sequence (NCBI Gene ID: 43740575) was amplified from an expression vector of SARS-CoV-2 N protein (Constructed by Dr. Wang Pei-Hui from Shandong university) and cloned into pB-TRE-EF1α-rtTA-2A-puroR-2A-EGFP using Clone Express II One Step Cloning Kit (Vazyme). The resultant plasmids were examined by sequencing (IGE Biotechnology).

8×10^5 H1 cells were co-transfected with pB-TRE-N-EF1α-rtTA-2A-puroR-2A-EGFP and pBase plasmids using Human Stem Cell Nucleofector® Kit (LONZA). The cells were plated in Matrigel-coated 24-well plates with 10 μM Y-27632 (Selleck) in mTeSR1 for 24 h, followed by selection in mTeSR1 medium with 300 ng /ml puromycin for 48 h, remaining cells were re-plated into 96-well plates for single-cell culture.

4.3. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde at RT for 15 min, washed with PBS, permeabilized, blocked with 10% FBS for 1 h. Primary antibodies were incubated for overnight at 4 °C, followed by secondary antibodies incubation for 1 h in the dark. Images were captured with confocal microscope (Zeiss 710 NLO). Antibodies used in this study are listed in Table 2.

4.4. Induction of SARS-CoV-2 N protein with doxycycline

WAe001-A-58 cells were plated at a density of $1.5 \times 10^4/cm^2$ on Matrigel-coated plates. 24 h after seeding, cells were treated with mTeSR1 medium supplemented with doxycycline for 48 h. The expression of SARS-CoV-2 N protein was verified by western blot. Antibodies used in this experiment are listed in Table 2.

4.5. Flow cytometry analysis

Cells were fixed in 4% paraformaldehyde for 15 min at RT, incubated with primary antibody for 30 min at 4 °C and followed by secondary antibody incubation for 30 min at 4 °C. The expression levels of cell surface markers were analyzed by Accuri C6 Plus flow cytometer (BD Biosciences). Antibodies used are listed in Table 2.

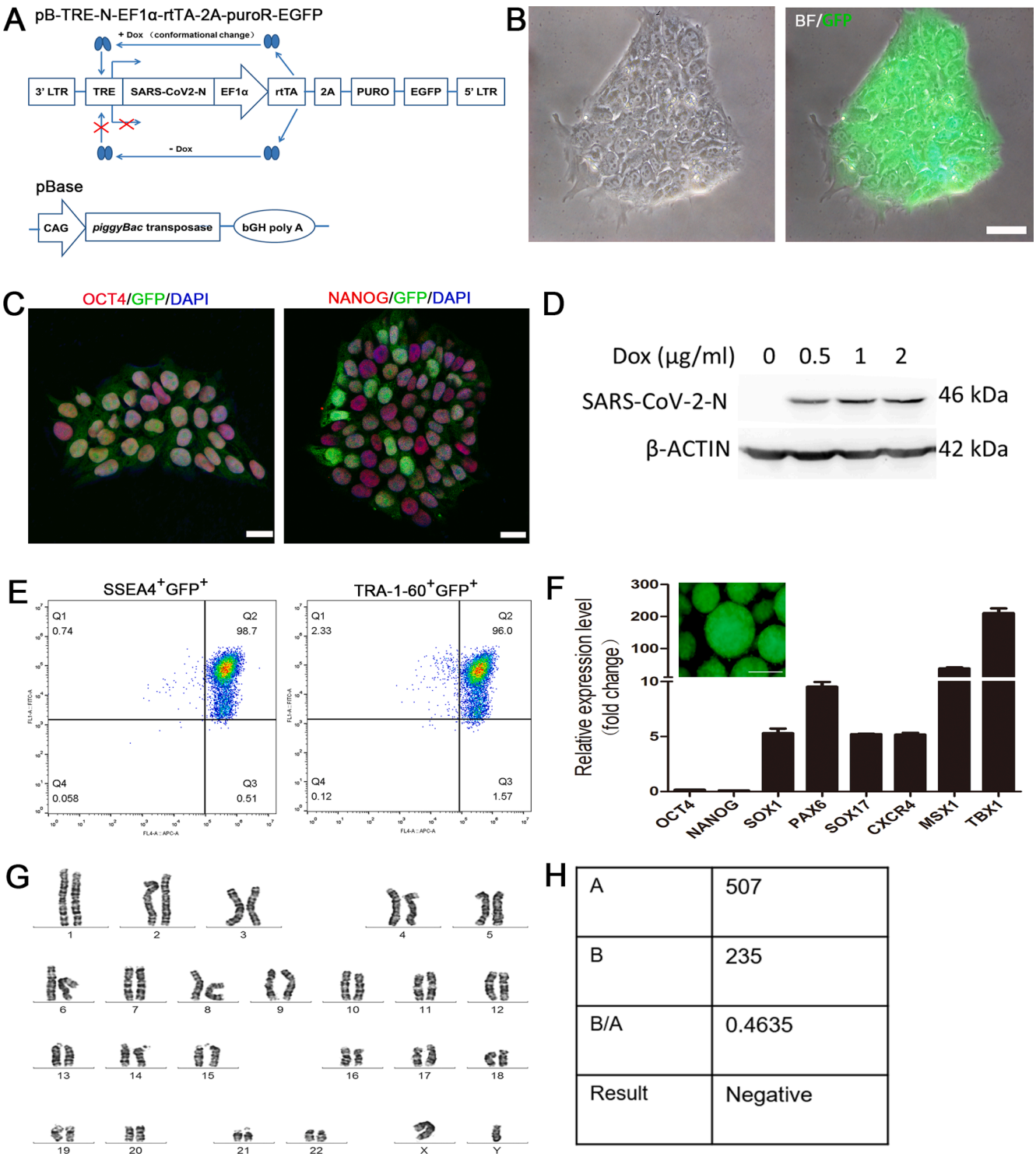

Fig. 1. Establishment and characterization of WAe001-A-58, an embryonic stem cell line with SARS-CoV-2 N protein doxycycline-inducible expression. (A) Schematic of the SARS-CoV-2 N protein doxycycline-inducible expression vector and the *piggyBac* system. (B) Phase contrast and green fluorescence images showed the morphology and GFP expression of WAe001-A-58 cells, scale bar 20 μm. (C) Immunofluorescence staining of pluripotent markers OCT4 (red) and NANOG (red), scale bar 20 μm. (D) Westernblot analysis showed SARS-CoV-2 N protein expression in different concentration of doxycycline treated WAe001-A-58 cells. (E) Flow cytometric analysis of SSEA4 and TRA-1-60. (F) qRT-PCR analysis of pluripotent and the three germ layers markers relative to WAe001-A-58-EB cells (Pluripotent: *OCT4* and *NANOG*; Ectoderm: *SOX1* and *PAX6*; Endoderm: *SOX17* and *CXCR4*; Mesoderm: *MSX1* and *TBX1*). (G) The karyotype analysis of WAe001-A-58 cell line. (H) Mycoplasma detection of WAe001-A-58 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Reagents details.

Antibodies for immunofluorescence, flow-cytometry and western blot			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Marker	Mouse anti-OCT4	1:200	Santa Cruz, Cat #: sc-5279 RRID: AB_628051
Pluripotency Marker	Mouse anti-NANOG	1:200	Santa Cruz Cat #: sc-293121 RRID: AB_2665475
Pluripotency Marker	Mouse anti-SSEA4	1:200	Abcam Cat#: ab16287 RRID: AB_778073
Pluripotency Marker	Mouse anti-TRA-1-60	1:200	Santa Cruz Cat#: sc-21705 RRID: AB_628385
SARS-CoV-2 Structural Protein	Rabbit anti-SARS-CoV-2 Nucleocapsid antibody	1:1000	Sino Biological Inc Cat#: 40143-R019 RRID: AB_2827973
House-Keeping Protein	Rabbit anti-β-Actin	1:1000	Cell Signaling Technology Cat#: 8457S RRID: AB_10950489
Secondary antibody	Donkey anti Mouse, Alexa Fluor 568	1:400	Thermo Fisher Scientific Cat #: A10037 RRID: AB_2534013
Secondary antibody	Donkey anti Mouse, APC-Alexa Fluor 750	1:400	Thermo Fisher Scientific Cat #: A-21006 RRID: AB_2535706
Secondary antibody	Goat anti-Rabbit IgG, HRP-linked Antibody	1:3000	Cell Signaling Technology Cat #: 7074S RRID: AB_2099233

Primers		
	Target	Forward/Reverse primer (5′-3′)
House-Keeping Gene (qPCR)	*β-Actin*	CTCCTCCTGAGCGCAAGTACTC/ TCCTGCTTGCTGATCCACATC
Pluripotency Marker (qPCR)	*OCT4*	CCTCACTTCACTGCACTGTA/ CAGGTTTTCTTTCCCTAGCT
Pluripotency Marker (qPCR)	*NANOG*	TGAACCTCAGCTACAAACAG/ TGGTGGTAGGAAGAGTAAAG
Ectoderm Marker (qPCR)	*SOX1*	TTTCCCCTCGCTTTCTCA/ TGCAGGCTGAATTCGGTT
Ectoderm Marker (qPCR)	*PAX6*	TTGCTTGGGAAATCCGAG/ TGCCCGTTCAACATCCTT
Endoderm Markers (qPCR)	*SOX17*	GCATGACTCCGGTGTGAATCT/ TCACACGTCAGGATAGTTGCAGT
Endoderm Markers (qPCR)	*CXCR4*	ACTACACCGAGGAAATGGGCT/ CCCACAATGCCAGTTAAGAAGA
Mesoderm Marker (qPCR)	*MSX1*	TCCGCAAACACAAGACGA/ ACTGCTTCTGGCGGAACTT
Mesoderm Marker (qPCR)	*TBX1*	ATGCTGCTCATGGACTTCG/ TTCGCGAAGGGATTGCT

4.6. Karyotype analysis

Cells were incubated with colcemid for 130 min, dissociated, incubated in hypotonic solution for 20 min and fixed in methanol: acetic acid (3:1). Chromosomes of metaphase were then classified according to the standard G-banding technique.

4.7. Embryonic body (EB) formation and in vitro differentiation

Cells were dissociated with collagenase IV (Gibco) for 30 min at 37 °C, resuspended in EB medium (DMEM/F12 (Gibco) supplemented with 20% knockout serum replacement (Gibco), 1% L-GlutaMax (Gibco), 1% NEAA (Gibco) and 0.1% β-mercaptoethanol (Gibco)) and cultured for 14 days.

4.8. Real-time PCR analysis

Total RNA was extracted with TriZol reagent according to manufacturer's instructions (Invitrogen). Real-time PCR analysis was conducted in triplicate on a CFX96^{TM} Real-Time System (Bio-Rad). Primers used are listed in table 2.

4.9. Mycoplasma detection

Mycoplasma detection was performed with MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza) according to the manufacturer's instructions.

4.10. STR analysis

STR analysis was carried out by IGE Biotechnology LTD (Guangzhou, China)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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