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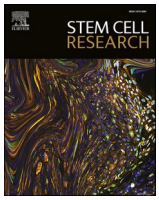

Lab Resource: Genetically-Modified Single Cell Line

Generation of a gene-edited H9 embryonic stem cell line carrying a DOX-inducible NGN2 expression cassette in the CLYBL locus

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A B S T R A C T

The pro-neural transcription factor neurogenin-2 (NGN2) possesses the ability to rapidly and effectively transform stem cells into fully operational neurons. Here we report the successful generation of a modified H9 human embryonic H9 stem cell line containing a doxycycline (DOX) inducible NGN2 expression construct featuring a floxed Blasticidin/mApple selection module in the safe-harbor locus CLYBL. This cell line retains its pluripotent state in the absence of DOX, yet readily transitions into a neuronal state upon DOX introduction.

Resource Table

Unique stem cell line identifier	UOWe010-A-W
Alternative name(s) of stem cell line	H9 NGN2 B6
Institution	University of Wollongong
Contact information of the reported cell line distributor	Sara Miellet, miellet@uow.edu.auMirella Dottori, mdottori@uow.edu.au
Type of cell line	Embryonic stem cell line
Origin	Human
Additional origin info	Age: blastocyst stage Sex: female, 46, XX Ethnicity if known: n/a
Cell Source	Human embryonic stem cell (hESC) line H9 (WA09) purchased from WiCell
Method of reprogramming	n/a
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	n/a
The cell culture system used	Feeder-free culture
Type of the Genetic Modification	Transgene insertion
Associated disease	n/a
Gene/locus	CLYBL insertion
Method of modification / user-customizable nuclease (UCN) used, the resource used for design optimization	CRISPR/Cas
User-customizable nuclease (UCN) delivery method	Lipid-mediated transfection of RNP
All double-stranded DNA genetic material molecules introduced into the cells	Homology directed repair (HDR) donor vector: Addgene #124229 (CLYBL-TO-hNGN2-BSD-mApple) Addgene #41856 (Non-integrating expression of mouse p53DD)

(*continued on next column*)

(*continued*)

Analysis of the nuclease-targeted allele status	Genotyping PCR: PCR for WT allele and junction PCR, followed by sequencing of junction PCR product
Method of the off-target nuclease activity prediction and surveillance	Not performed
Descriptive name of the transgene	Dox-inducible *NGN2* insertion with floxed Blasticidin resistance and nuclear mApple
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	Blasticidin
Inducible/constitutive expression system details	TET or DOX inducible system
Date archived/stock creation date	May 2020
Cell line repository/bank	https://hpscreg.eu/cell-line/WAe009-A-W
Ethical/GMO work approvals	University of Wollongong Health and Medical Human Research Ethics Committee, Approval # 2020/451University of Wollongong Institutional Biosafety Committee, Approval # GT19/08
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	CLYBL-TO-hNGN2-BSD-mApple was a gift from Michael Ward (Addgene plasmid # 124229; https://n2t.net/addgene:124229; RRID: Addgene_124229)

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1. Resource utility

The acceleration of neuronal differentiation from induced pluripotent stem cells (iPSCs) has been demonstrated through the expression of neurogenin 2 (NGN2) (Zhang et al., 2013; Hulme et al., 2022). Here, we present a H9 human embryonic stem cell line (hESC) harbouring a stably integrated doxycycline-inducible *NGN2* transgene within the CLYBL locus. Overexpression of NGN2 facilitates the rapid, effective, and scalable generation of neurons derived from pluripotent stem cells. Table 1

2. Resource details

The H9 NGN2 B6 cell line was generated by stably integrating a gene cassette in the CLYBL safe-harbour locus of the established H9 hESC cell line (Thomson et al., 1998 6). The gene cassette contains a Neurogenin-2 gene under a doxycycline-inducible promoter, a reverse TET transactivator (TRE3G) under the CAG promoter as well as a nuclear mApple gene and a Blasticidin S deaminase gene (BSD) under the EF-1α promoter (Fig. 1A). We used CRISPR/Cas9 homology-directed repair (HDR) for stable integration. After selection with Blasticidin, a monoclonal H9 NGN2 B6 cell line was produced. The monoallelic insertion of the gene cassette in the CLYBL locus was confirmed via junction PCR (Fig. 1B, E). The functionality of the inducible *NGN2* insertion was verified through the application of DOX for 2 days, resulting in the transformation cells exhibiting a characteristic neuronal morphology within 6 days (Fig. 1C). NGN2 overexpression was confirmed via immunostaining on day 3 after DOX induction and RT-qPCR 48hrs after DOX induction (Fig. 1C, D). Under uninduced conditions (-DOX), there was negligible (Cq $>$ 35) mRNA expression of *NGN2* (Fig. 1D) and no NGN2 expression was detected via immunostaining (Fig. 1H). H9 NGN2 B6 showed typical pluripotent stem cell morphology assessed by brightfield microscopy (Fig. 1G left panel) and stained positive for the pluripotency markers OCT4 and SOX2 via immunocytochemistry (Fig. 1G). A normal karyotype was observed at passage 5 (Fig. 1F). Spontaneously differentiating embryoid bodies of H9 NGN2 B6 showed the ability to differentiate into derivatives of the three germ layers. Positive staining for mesoderm marker alpha-smooth muscle actin,

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal Morphology	Fig. 1 panel G
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	H9 NGN2 B6 p12 + 5 positive for OCT4 and SOX2	Fig. 1 panel G
	Quantitative analysis (i.e. Flow cytometry, RT-qPCR)	Not performed	
Karyotype	G-banding	H9 NGN2 B6 p3 + 2 + 2 is 46XX (15 cells, resolution 400 bhps)	Fig. 1 panel F
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	Junction PCR confirms heterozygous integration at the CLYBL locus, followed by sequencing of junction PCR	Fig. 1 panel B and E
	Evaluation of the - (homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	See above	
	Transgene-specific PCR (when applicable)	See above (junction PCR)	
Verification of the absence of random plasmid integration events	PCR/Southern	Not performed	
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	STR profile tested for 16 sites of H9 NGN2 B6 p10	Supplementary File
		D5S818	100 % match with WA09/H9
		D13S317 D7S820 D16S539 vWA TH01 Amel TPOX CSF1PO D8S1179 D21S11 D3S1358 D2S1338 D19S433 D18S51 FGA	
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Sequencing of junction PCR product	Fig. 1 panel E
	PCR-based analyses	Insertion transgenes into one allele of CLYBL tested by junction PCR	Fig. 1 panel B
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	Not performed	
Off-target nuclease activity analysis	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	Not performed	
Specific pathogen-free status	Mycoplasma	Mycoplasma testing of H9 NGN2 B6 p12 + 4 + 3 by luminescence: no contamination present	Supplementary File
Multilineage differentiation potential	Embryoid body formation	Three germ layer formation: germ layer specific gene expression verified by Immunostaining	Fig. 1I
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	n/a	
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	n/a	
	HLA tissue typing	n/a	

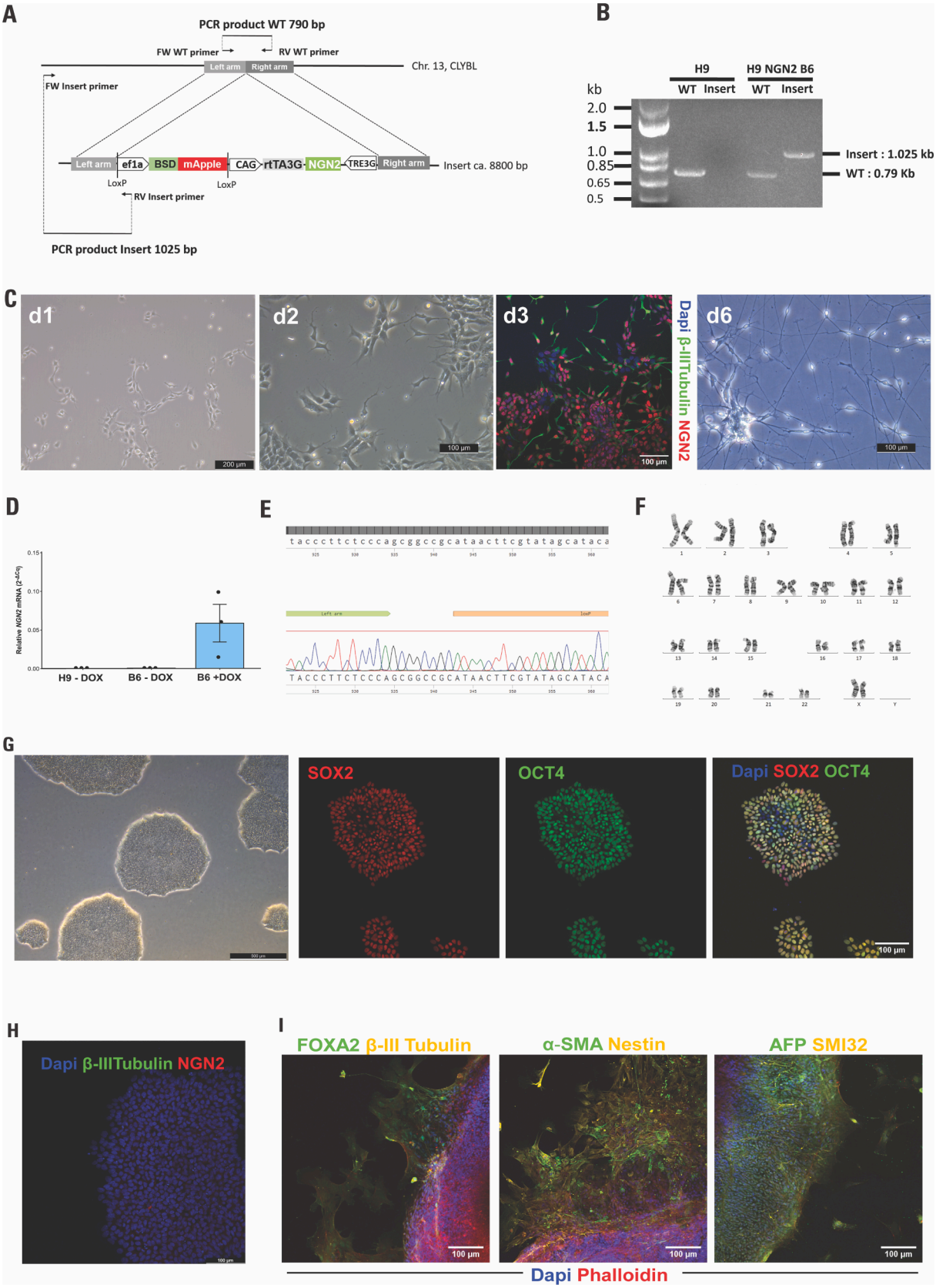
A
PCR product WT 790 bp
FW WT primer
RV WT primer
Chr. 13, CLYBL
Left arm
Right arm
FW Insert primer
Left arm
ef1a
BSD
mApple
CAG
rtTA3G
NGN2
TRE3G
Right arm
Insert ca. 8800 bp
LoxP
LoxP
RV Insert primer
PCR product Insert 1025 bp
B
H9
H9 NGN2 B6
WT
Insert
WT
Insert
kb
2.0
1.5
1.0
0.85
0.65
0.5
Insert : 1.025 kb
WT : 0.79 Kb
C
d1
d2
d3
Dapi β-IIITubulin NGN2
d6
200 µm
100 µm
100 µm
100 µm
D
Relative NGN2 mRNA (2^-ΔCq)
0.15
0.10
0.05
0.00
H9 - DOX
B6 - DOX
B6 +DOX
E
t a c c c t t c t c c c a g c g g c c g c a t a a c t t c g t a t a g c a t a c a
T A C C C T T C T C C C A G C G G C C G C A T A A C T T C G T A T A G C A T A C A
F
G
SOX2
OCT4
Dapi SOX2 OCT4
100 µm
H
Dapi β-IIITubulin NGN2
I
FOXA2 β-III Tubulin
α-SMA Nestin
AFP SMI32
100 µm
100 µm
100 µm
Dapi Phalloidin

Fig. 1.

ectoderm markers beta-III tubulin, SMI32 and Nestin as well as endoderm markers FOXA2 and alpha-Fetoprotein was confirmed via immunofluorescence staining (Fig. 1I). H9 NGN2 B6 was genetically matched to the original H9 line via STR profiling.

3. Materials and methods

3.1. Cell culture

H9 and H9 NGN2 B6 were cultured on vitronectin (10 μg/ml) coated plates in E8 media (StemCell Technologies) and passaged using EDTA when approximately 80 % confluency was reached. The cells were incubated at 37 °C in a humidified incubator at 5 % CO_2 and 20 % O_2.

3.2. Gene editing

Prior to gene editing, cells were dissociated using Accutase and plated at 0.03 x 10^6 per cm^2 in E8 supplemented with small molecule, Y27632 (10 μM). CRISPR/Cas9 components targeting the CLYBL region were delivered as a ribonucleoprotein (RNP) complex, which consisted of the CRISPR/Cas9 guide RNA (crRNA:tracrRNA duplex) and a high-fidelity Cas9 nuclease protein (IDT). A total of 1 x 10^6 cells were transfected with the RNP complex, 2 μg of the HDR donor plasmid (Addgene plasmid 124,229 CLYBL-TO-hNGN2-BSD-mApple) and 0.4 μg of the Addgene plasmid #41856 (non-integrating expression of mouse p53DD to facilitate integration via HDR) via Lipofectamine Stem Transfection reagent (Invitrogen). After 2 days cells were treated with 10 μg/ml Blasticidin to enrich for cells containing the gene cassette. Single cell colonies were then produced by limited dilution.

3.3. PCR

Genomic DNA was extracted using the PureLink genomic DNA Mini Kit (Thermofisher). PCR was performed using the Invitrogen Platinum Green Hot Start PCR 2x Master Mix using the primers listed in Table 2 and run on an Eppendorf Mastercycler Pro. Messenger RNA (mRNA) was extracted using the PureLink RNA Mini Kit (ThermoFisher Scientific) and converted to cDNA using the iScript™ gDNA Clear cDNA Synthesis Kit (Biorad) according to the manufacturer instructions. Amplification of cDNA was performed on the Quantstudio™ 5 Real-Time PCR System using KiCqStart (Sigma) Primers listed in Table 2. Results were normalised to GAPDH, β2M, and PPIA housekeeper genes. Expression is given as relative mRNA (fold-change). Each sample gene expression was calculated using the 2^–ΔCq method, whereby ΔCq is given by: ΔCq = Cq (*NGN2*) – ΔCq (Average of housekeeping genes).

3.4. Immunofluorescence analysis

Cells were fixed with 4 % paraformaldehyde for 10 min at room temperature (RT), permeabilized in 0.1 % Triton-x 100 for 10 min at RT and blocked in 10 % donkey serum in phosphate-buffered saline (PBS), followed by overnight primary antibody incubation in 5 % donkey serum in PBS at 4 °C. Secondary antibodies were diluted in 5 % donkey serum and incubated for 2 h at RT. Nuclei were stained with DAPI at 1 μg/ml. The antibodies that were used are listed in Table 2.

3.5. Embryoid body formation

Cells were lifted with EDTA, pelleted, re-suspended in embryoid body (EB) medium (Knockout DMEM/F12, 2 mM Glutamax, 20 % Knockout serum, 0.1 mM non-essential amino acids (Thermofisher), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich)), transferred to ultra-low attachment plates and placed at 37 °C in a humidified incubator at 5 % CO_2 and 20 % O_2. After 4 days, embryoid bodies were plated onto Matrigel in EB medium for 17 days and then processed for immunostaining.

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	mouse anti-OCT4 goat anti-SOX2	1:200 1:200	Santacruz, sc-5279 R and D, RDSAF2018SP
Differentiation Markers	mouse anti-alpha-Smooth muscle actin mouse anti-alpha-Fetoprotein rabbit anti-Nestin rabbit anti-SMI32 mouse anti-FOXA2 rabbit anti-beta-III-tubulin	1:200 1:200 1:200 1:200	Sigma-Aldrich, CBL171 Sigma-Aldrich, 200,418 Sigma-Aldrich, ABD69 Abcam, ab8135
		1:500	Abcam, ab60721
Dox test	mouse anti-beta-III-tubulin	1:500	Millipore, MAB1637
	rabbit anti-Neurogenin-2	1:250	Thermofischer, PA5-78556
Secondary antibodies	Anti-mouse alexa fluor 488, donkey	1:500	Abcam, ab150109
	Anti-rabbit alexa fluor 555, donkey	1:500	Abcam, ab150062
	Anti-rabbit alexa fluor 647, donkey	1:500	Abcam, ab150063
Nuclear stain	DAPI	1 μg/mL	D9542, Sigma-Aldrich
Site-specific nuclease			
Nuclease information	Nuclease type/version	IDT SpCas9 Hi-Fi v.3	
Delivery method	Lipofection	Lipofectamine™ Stem Transfection Reagent (Invitrogen)	
Selection/ enrichment strategy	PCR/expression of Blasticidin resistance gene		
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
Genotyping	FW WT primer	TGACTAAACACTGTGCCCCA	
	RV WT primer	AGGCAGGATGAATTGGTGGA	
	FW Insert primer	CAGACAAGTCAGTAGGGCCA	
	RV insert primer	AGAAGACTTCCTCTGCCCTC	
RT qPCR	FW NGN2	AAGATGTTCGTCAAATCCG	
	RV NGN2	CTCCTCCTCCTCTTCTTC	
	FW GAPDH	TCGGAGTCAACGGATTTGGT	
	RV GAPDH	TTCCCGTTCTCAGCCTTGAC	
	FW β2M	AAGGACTGGTCTTTCTATCTC	
	RV β2M	GATCCCACTTAACTATCTTGG	
	FW PPIA	ACGTGGTATAAAAGGGGCGG	
	RV PPIA	CTGCAAACAGCTCAAAGGAGAC	
sgRNA/crRNA sequence including PAM sequence (bold)	CLYBL CRISPR sgRNA	ATGTTGGAAGGATGAGGAAA **TGG**	
Genomic target sequence	For CLYBL CRISPR sgRNA	TTTCCTCATCCTTCCAACAT	

3.6. Dox-test

Cells were passaged onto vitronectin in E8. After one day (Day1), media was changed to Neurobasal™ medium supplemented with 1 % N2 and 1 % B-27 minus vitamin A, 1 % IST-A, 1 % L-glutamine

(Thermofisher) and 2 μg/ml doxycycline (Sigma-Aldrich). After 3 days, doxycycline was withdrawn from the medium. Cell morphology was monitored using brightfield microscopy and cell were fixed and stained for beta-III tubulin and NGN2 on day 3 (Immunofluorescence analysis and Table 2 for antibody details). To determine leaky gene expression of *NGN2* in an uninduced state, RNA was extracted from H9 NGN2 B6 cells with DOX, H9 NGN2 B6 cells without DOX, as well as unmodified H9 cells without DOX.

3.7. Karyotype analysis

Karyotype analysis was performed in passage 3 + 2 + 2 in the cytogenetics laboratory of Sullivan Nicolaides Pathology.

CRediT authorship contribution statement

Sara Miellet: Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Formal analysis, Writing – original draft, Visualization, Data curation. **Mitchell St Clair-Glover:** Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. **Marnie Maddock:** Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. **Mirella Dottori:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mirella Dottori reports financial support was provided by Freidreich's Ataxia Research Alliance (FARA). Mirella Dottori reports financial support was provided by Friedrich Ataxia Research Association. Mirella Dottori reports financial support was provided by Australian Research Council. Mirella Dottori reports financial support was provided by Medical Research Future Fund Stem Cells Mission. M Dottori has collaborated with Dr Dmitry A. Ovchinnikov, who is an Editor for Stem Cell Research. If there are other authors, they 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2024.103312.

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