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Lab resource: Stem Cell Line

# Generation of *GADD45A* gene knockout human embryonic stem cell line using CRISPR/Cas9

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A B S T R A C T

*GADD45A* is a DNA damage and stressful growth arrest inducible protein, also it is shown to a be tumor suppressor gene and a chromatin relaxer associated with opening chromatin during the somatic reprogramming. However, its role in human embryonic stem cells and human embryonic stem cell modeled development has been merely documented. To illustrate the function of *GADD45A* in the human embryonic stem cell biology, we reported a *GADD45A* knockout human embryonic stem cell line by CRISPR/Cas9 mediated gene targeting. This cell line displayed normal karyotype, pluripotent stem cell marker expression and differentiation potential both in vivo and vitro.

## 1. Resource table

| | |
|---|---|
| Unique stem cell lines identifier | *GIBHe011-A* |
| Alternative names of stem cell lines | *WA01 -GADD45A-Knockout* |
| Institution | *Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences* |
| Contact information of distributor | Ke Huang,huang_ke@gibh.ac.cn; Yichu Nie, nieych3@mail.sysu.edu.cn |
| Type of cell lines | *ESC* |
| Origin | *human* |
| Cell Source | *blastocyst* |
| Clonality | *Clonal* |
| Method of reprogramming | *N/A* |
| Gene modification | *YES* |
| Type of modification | *Induced mutation* |
| Associated disease | *N/A* |
| Gene/locus | *GADD45A/1p31.3* |
| Method of modification | *CRISPR/Cas9* |
| Name of transgene or resistance | *N/A* |
| Inducible/constitutive system | *N/A* |
| Date archived/stock date | *August 3,2020* |
| Cell line repository/bank | *N/A* |
| Ethical approval | *Cell line was used according to institutional guidelines* |

## 2. Resource utility

The *GADD45A* homologous gene knockout human embryonic stem cells (hESCs) could be used to explore the exact role of *GADD45A* in both hESCs and hESC modeled development, which might give insights into the regulation network of epigenetics, cell cycle and pluripotency. Also, this cell line could be used in disease modeling with genetic disorder of *GADD45A* (Chen et al., 2016).

## 3. Resource details

The WA01 *GADD45A* knockout cell lines were produced by

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disrupting the exon 2 through CRISPR/Cas9 mediated gene editing from WA01 human embryonic stem cells (hESCs) (Fig. 1A). The methods for gene mutation in hESCs were described in Materials and Methods section. The mutated clones were picked and confirmed by genomic PCR and following Sanger sequencing. We observed a mutated clone WA01 -*GADD45A*with 7 and 2 nucleobase deletion in each allele (Fig. 1B). These mutations resulted in the formation of premature stop code in 75th/52th amino acids (aa) of the *GADD45A* protein, while the wild-type protein has 165 aa (Table S1). Also, the truncation of the GADD45A protein could lead to the damage of its conserved domain (located from 21th to 123th aa), which is crucial for the normal function of GADD45A. In addition, the expression level of the *GADD45A* was further confirmed by RT-qPCR, which indicated the absent of *GADD45A* expression in the mutated cell line and the successful establishment of WA01-*GADD45A*$^{-/-}$ cell line (Fig. S1A).

Despite the genetic mutation in the *GADD45A* loci, the WA01*GADD45A*$^{-/-}$ cell line showed normal embryonic stem cell morphology (Fig. S1B), karyotype (Figure C) and classical pluripotent maker expression, such as OCT4, SSEA4, TRA-1-60 and TRA-1-81 (Fig. 1D and E). Also, the mutated cell line remained the ability to differentiate into three germ layers, highlighting its differentiation potential both in vitro and in vivo, as demonstrated by embryoid body differentiation and teratoma formation (Fig. 1F and G). Next, the cell line also showed no off-target after examining the potential off-target sites, and was found to be free of mycoplasma contamination. Furthermore, this cell line was confirmed to be identical to the WT WA01 cell line through short tandem repeat (STR) analysis. The above information of the WA01-*GADD45A*$^{-/-}$ cell line were summarized in Resource Table and Table 1.

## 4. Materials and methods

### 4.1. Cell culture

The WA01 and WA01-*GADD45A*$^{-/-}$ human embryonic stem cell line were maintained in mTeSR1 medium (Stemcell Technologies) on matrigel coated plates (Corning), at 37 °C and with 5% $CO_2$. The hESCs were passaged every 3–4 days with accutase (Sigma).

### 4.2. Gene targeting

About 2 μg of the pX459 (Ran et al., 2013) vector, containing the sgRNA targeting the exon 2 of *GADD45A* gene designed by sgRNA Designer (Doench et al., 2016), (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), were electroporated into 1 × $10^6$ WA01 hESCs, using Nucleofector™ 2b Device (Lonza). Particularly, the pX459 plasmid was obtained as a gift from Feng Zhang (Addgene plasmid # 62988; http://n2t.net/addgene:62988; RRID: Addgene_62988) by Dr. Hung-Fat Tse's lab. Then, the cells were seeded onto 3 well of the matrigel-coated 6-well plate in the presence of ROCK inhibitor – Y27632 (5 μM, Sigma). After 48 h, the cells were selected with puromycin (0.5 μg/mL) for 24 h. About one week later, the individual clones were picked for further screening. Initially, the genomic PCR using primer set F/R, (Fig. 1A) and Sanger sequencing were used to analyze the gene mutation around the sgRNA targeting site. In specific, the PCR products were purified and ligated with pCE2-TA-Blunt-Zero cloning vector (Vazyme). Then, the ligation products were further transformed into DH5α Competent Cells. After transformation, independent clones were picked for Sanger sequencing to analyze the genotype of the mutated clone and the Sanger sequencing tracks of the wild-type and mutated clone were viewed by SnapGene software. Then, the *GADD45A* expression of the mutated clone was verified by the RT-qPCR. In this case, the forward primer for the RT-qPCR was located on the exon 1 and the reverse primer was on the exon 2 with the 3′ end of the primer at the mutated site. The genomic PCR (F/R) and RT-qPCR (qF/qR) primers used in this study were marked on Fig. 1A and listed in Table 2.

### 4.3. RNA isolation and RT-qPCR

Total RNA was isolated by the TRIzol (Sigma) following the manufacturer's instruction. Then, the RNA was reverse-transcribed into cDNA using the HiScript® III RT SuperMix for RT-qPCR (Vazyme). The RT-qPCR were performed in triplicate on a CFX96 machine (Bio-Rad) using the SYBRGreen Mix (Vazyme), under following conditions: initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. The primer set qF/qR were marked on Fig. 1A and listed in Table 2.

### 4.4. Flow cytometric analysis

The cells were digested into single cells and suspend in Cytofix/Cytoperm™ Fixation (BD) solution at room temperature for 15 min, then permeabilized in the Permeabilization solution (BD) for at least 10 min. After permeabilization, single-cell suspensions were incubated with the primary antibody for 30 min at 4 °C, and subsequently with the secondary antibody (Alexa Fluor 488 Goat anti-Mouse IgG) for addition 30 min at 4 °C. After the incubation, the expression of the pluripotent markers in this study were analyzed by the CytoFLEX Flow Cytometer (Beckman). The antibodies were listed in Table 2.

### 4.5. Immunostaining

In brief, the hESCs maintained on the 24-well-plate were washed with phosphate buffer solution (PBS) and fixed with 4% paraformaldehyde at room temperature for 2 min. Cells were further washed with PBS and incubated with primary antibody in PBS with 10% bovine serum albumin (BSA), 0.3% TritonX-100 overnight at 4 °C. Then, the cells were washed twice and further staining with secondary antibody (Alexa Fluor 488 Goat anti-Mouse IgG) for 1 h in PBS with 1% BSA at room temperature. After the incubation, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for additional 5 min. After this procedure, the cells were washed with PBS twice and the images were captured with Confocal laser scanning microscope (Zeiss LSM 710). The antibodies were listed in Table 2.

### 4.6. Karyotype analysis

The hESCs at 70–80% confluency were treated with colcemid for 130 min. Then, the hESCs were harvested and treated with a hypotonic solution for further karyotype analysis. Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature according to the standard G-banding technique.

### 4.7. Teratoma formation and analysis

The teratomas formation implicating animal research have been reviewed and approved by IACUC at GIBH. About 1 × $10^6$ WA01 hESCs were resuspended in 30% matrigel in cold DMEM/F12 (Hyclone), and injected into immuno-deficient NOD-SCID mice (Vital River). The teratomas were harvested after 6 weeks and fixed in 4% paraformaldehyde, and further stained with hematoxylin/eosin (H&E).

### 4.8. Embryoid body (EB) differentiation

The EBs were formed by dissociation the hESCs clones with Accutase (sigma) into single cells and then suspended in the mTeSR1 medium (Stemcell Technologies) in the presence of Y-27632 (5 μM, Sigma). After 24 h, the EBs were formed, and then maintained in EB medium (DMEM/F12 + 20% knockout serum replacement (KSR; Gibco) + 1% L-GlutaMAX (Gibco) + 1% NEAA (Gibco) + 0.1% β-mercaptoethanol (Gibco)) for 12 days. Then, the cells were collected for analysis.

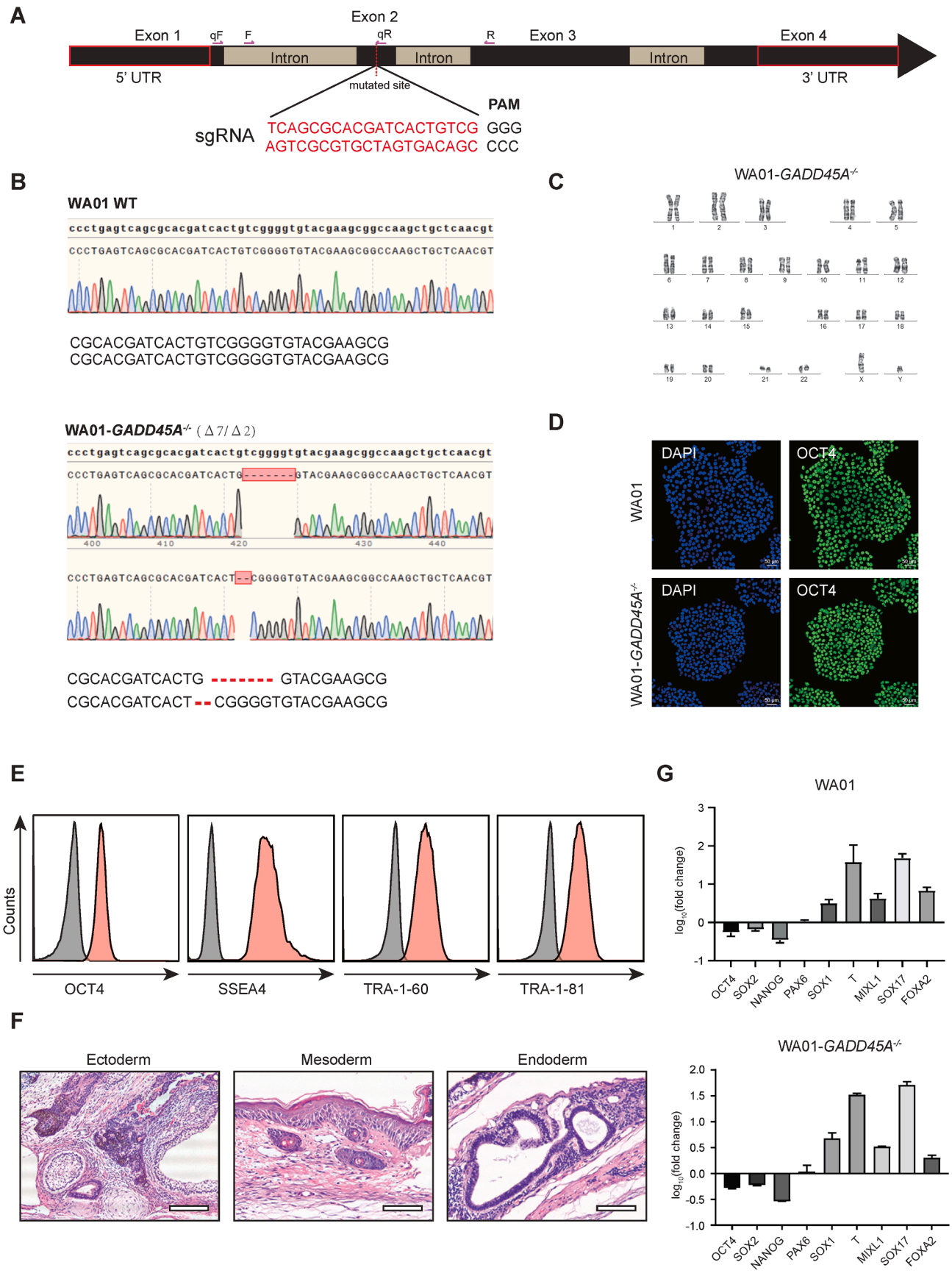


**Fig. 1.** Generation of GADD45A gene knockout human embryonic stem cell line.

**Table 1**
Characterization and validation. Detailed instructions for identification of *GADD45A* knockout human embryonic stem cell line.

| Classification | Test | Result | Data |
|---|---|---|---|
| Morphology | Photography | Normal | Fig. S1 panel C |
| Phenotype | Flow cytometry | OCT4, SSEA, TRA-1–60, TRA-1–81 positive | Fig. 1 panel B |
| | Immunofluorescence | OCT4 positive | Fig. S1 panel E |
| Genotype | Karyotype(G-banding) and resolution | 46XY | Fig. S1 panel D |
| Identity | STR analysis | 21 sites tested and 100% matched with the WA01 hESC cell line | Table S1 |
| Mutation analysis | Sequencing | Heterozygous knockout | Fig. 1 panel A |
| | Southern Blot OR WGS | N/A | |
| Microbiology and virology | Mycoplasma | Negative | Data available with authors |
| Differentiation potential | Embryoid body formation | Down-regulated genes Pluripotency Markers: OCT4, SOX2, NANOG Up-regulated genes: Ectoderm Marker: PAX6, SOX1 Mesoderm Marker: T, MIXL1 Endoderm Marker: SOX17, FOXA2 | Fig. 1 panel C |
| | Teratoma | Formation of three germ layer | Fig. 1 panel D |
| Donor screening (optional) | HIV 1 + 2 Hepatitis B, Hepatitis C | N/A | N/A |
| Genotype additional info (optional) | Blood group genotyping | N/A | N/A |
| | HLA tissue typing | N/A | N/A |

### 4.9. Off-target analysis

The potential off-target sites (POTs) of the sgRNA were predicted using the CCTop - CRISPR/Cas9 target online predictor (https://cctop.cos.uni-heidelberg.de) (Stemmer et al. 2015). The genomic PCR products of the POTs were confirmed by Sanger sequencing. The primers were listed in Table 2 and the results were listed in the Fig. S1C.

### 4.10. Detection of mycoplasma contamination

Mycoplasma was detected by MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza) under the guide of the manufacturer's instruction. Briefly, a total of 1 ml cell supernatant was taken and centrifuged at 250*g* for 5 min to remove the cell debris for the test. To measure the A value, 100 μl supernatant was mixed with the same volume of MycoAlert PLUS Reagent in luminometer plate and the A value was read after 5 min incubation. Similarly for the B value, 100 μl of the supernatant was added to the same value of MycoAlert™ PLUS Substrate and the B value was measured after 10 min incubation. Finally, the ratio of (B/A) were calculated. If the ratio was small than 1, indicating no mycoplasma contamination in the sample. Data are available with the authors upon reasonable request.

### 4.11. Short tandem repeat (STR) analysis

STR analysis was performed with detection of 21 loci by IGEbio Co.,

**Table 2**
Reagents details.

| Antibodies used for immunocytochemistry/flow-cytometry | | | |
|---|---|---|---|
| | Antibody | Dilution | Company Cat # and RRID |
| Pluripotency marker | Mouse anti-SSEA-4 | 1:100 | Santa Cruz Biotechnology Cat# sc21704, RRID: AB_628289 |
| Pluripotency marker | Alexa Fluor® 647 Mouse anti-TRA-1-60 | 1:100 | BD biosciences Cat# 560850, RRID:AB_10565983 |
| Pluripotency marker | Alexa Fluor® 647 Mouse anti-Oct3/4 | 1:100 | BD biosciences Cat# 560307, RRID: AB_1645319 |
| Pluripotency marker | Alexa Fluor® 647 Mouse anti-TRA-1-81 | 1:100 | BD biosciences Cat# 560793, RRID: AB_10550550 |
| Secondary antibody | Alexa Fluor 488 Goat anti-Mouse IgG | 1:500 | Thermo Fisher Scientific Cat# A-11001, RRID: AB_2534069 |
| Secondary antibody | Alexa Fluor 647 Goat anti-Mouse IgG | 1:200 | Invitrogen Cat# A-21235, RRID:AB_2535804 |

| Primers | | |
|---|---|---|
| | Target | Forward/Reverse primer (5′-3′) |
| Targeted gene (PCR, F/R) | GADD45A | CTAAAAAGTTTGCACAGGGCAACTCCC/ GTTCTCGCAGCAAAACGCCTGGAT |
| Targeted gene (qPCR, qF/qR) | GADD45A | TCGGCTGGAGAGCAGAAGAC/ CGCTTCGTACACCCCGAC |
| House-keeping gene (qPCR) | GAPDH | GGAGCGAGATCCCTCCAAAAT/ GGCTGTTGTCATACTTCTCATGG |
| Pluripotency marker (qPCR) | OCT4 | CCTCACTTCACTGCACTGTA/ CAGGTTTTCTTTCCCTAGCT |
| Pluripotency marker (qPCR) | SOX2 | CCCAGCAGACTTCACATGT/ CCTCCCATTTCCCTCGTTTT |
| Pluripotency marker (qPCR) | NANOG | TGAACCTCAGCTACAAACAG/ TGGTGGTAGGAAGAGTAAAG |
| Pluripotency marker (qPCR) | PAX6 | ATGTGTGAGTAAAATTCTGGGCA/ GCTTACAACTTCTGGAGTCGCTA |
| Pluripotency marker (qPCR) | SOX1 | AATTTTATTTTCGGCGTTGC/ TGGGCTCTGTCTCTTAAATTTGT |
| Pluripotency marker (qPCR) | T | TATGAGCCTCGAATCCACATAGT/ CCTCGTTCTGATAAGCAGTCAC |
| Pluripotency marker (qPCR) | MIXL1 | GGCGTCAGAGTGGGAAATCC/ GGCAGGCAGTTCACATCTACC |
| Pluripotency marker (qPCR) | SOX17 | CGCACGGAATTTGAACAGTA/ GGATCAGGGACCTGTCACAC |
| Pluripotency marker (qPCR) | FOXA2 | ACTACCCCGGCTACGGTTC/ AGGCCCGTTTTGTTCGTGA |
| Potential off-target sequencing | | |
| Potential off-target (POT) sequencing for sgRNA | POT1 | GAGACAATCTTGCCGAGCTTCAGTTTCC/ TTTGGGAAGACTGGGCATAAGCGGA |
| Potential off-target (POT) sequencing for sgRNA | POT2 | CATGGGCATTTCTTGGTCTTCCTCCTAT/ CTCTGCCAATTTGAGCTCCGGGTC |
| Potential off-target (POT) sequencing for sgRNA | POT3 | GGCAGGGTCTCCACAAATGGTTAGGG/ CAGAAGAGGGCAGGAGTGTGGGAAAT |
| Potential off-target (POT) sequencing for sgRNA | POT4 | TGTCTGGAGCTAGGGAGGGCTGTGTT/ TTTAGAGTTTTCCTGGCAGCAATGAGAGC |
| Potential off-target (POT) | POT5 | TGTCTTTCCATCCGCAGGTATCCGT/ GGTGCTGAGATGGTAAACCTGGCTGC |

(*continued on next page*)

**Table 2** (*continued*)

| Primers | | |
|---|---|---|
| sequencing for sgRNA | | |
| Potential off-target (POT) sequencing for sgRNA | POT6 | CAAAGTGTGTGGCAGGGGGAGAAAGTAT/ AACCAAGGCAGTTTTGCTCAGAGACCAT |
| Potential off-target (POT) sequencing for sgRNA | POT7 | CAATGTCCCTTTATAGAAAAACCTGGCAAAC/ CAAATCCAAGCCTGCCCTTCCAAAG |
| gRNA clone | GADD45A | caccgTCAGCGCACGATCACTGTCG aaacCGACAGTGATCGTGCGCTGA |

Ltd (Guangzhou, China).

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## 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.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.102090.

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