Generation of a genetically modified human embryonic stem cells expressing fluorescence tagged ATOX1

Meng-Hsuan Wen, Xihong Xie, Jian Tu, Dung-Fang Lee, Tai-Yen Chen

A B S T R A C T

ATOX1 is a copper chaperone involved in intracellular copper homeostasis, cell proliferation, and tumor progression. To investigate the physiologically relevant molecular mechanism of ATOX1 by using imaging-based approaches, we genetically modified ATOX1 in H1 hESCs to express mCherry-ATOX1 fusion protein under endogenous regulatory machinery. The fluorescence engineered hESC clone maintains characteristic stem cell features and can differentiate to all three germ layers, serving as a unique tool to dissect the role of ATOX1 in various cellular processes.

Resource Table

| Unique stem cell lines identifier | WAe001-A-29 |
|-----------------------------------|------------|
| Alternative names of stem cells  | H1_mCh-ATOX1 (KI) / UHMCe002-A-29 |
| Institution                       | University of Houston, Houston, TX, United States |
| Contact information of distributor| Tai-Yen Chen (tchen37@central.uh.edu) |
| Type of cell lines               | ESC |
| Origin                            | Human embryonic stem cell line |
| Additional origin info            | Age: N/A; Sex: Male Ethnicity if known: N/A |
| Cell Source                       | H1 hESCs |
| Clonality                         | Clonal |
| Method of reprogramming           | N/A |
| Gene modification                 | Yes |
| Type of modification              | Transgene expression (fluorescence protein) |
| Associated disease                | N/A |
| Gene/locus                        | 5q33.1; ATOX1 exon 2 |
| Method of modification            | CRISPR |
| Name of transgene or resistance   | mCherry |
| Inducible/constitutive system     | N/A |
| Date archived/stock date          | 5/18/2019 |

Cell line repository/bank: https://hpscreg.eu/cell-line/WAe001-A-29
Ethical approval: Cell lines were used according to institutional guidelines. UTHealth approval number: SCRO–16–01

1. Resource utility

We have engineered the ATOX1 exon 2 in a H1 human embryonic stem cell line to express mCherry-ATOX1 fusion proteins. This cell line can differentiate to different germ layers, which is useful for imaging-based studies on molecular mechanisms of ATOX1.

2. Resource details

ATOX1 is an intracellular copper chaperone, a cell-cycle modulator, and a therapeutic cancer target (Celauro, Mukaj, Fierro-Gonzalez, and Wittung-Stafshede, 2017; Hamza et al., 2001; Matson Dzebo, M., S., S., and E., 2018). ATOX1 deficiency results in perturbed intracellular trafficking of copper transporters and altered cell proliferation during early development. ATOX1 is ubiquitously observed in different cells and tissues. However the detail intracellular molecular behaviors, such as subcellular distribution and interaction dynamics, of ATOX1 in response to environmental cues are still unclear. To dissect the roles of ATOX1 in different cell contexts under endogenous regulatory by using...
Fig. 1.
Table 1
Characterization and validation.

| Classification          | Test                              | Result                                      | Data                      |
|-------------------------|-----------------------------------|---------------------------------------------|---------------------------|
| Morphology              | Photography                       | Normal                                      | Fig. 1 panel C            |
| Genotype                | Qualitative analysis: Immunocytochemistry | Positive staining for pluripotency factors NANOG, OCT3, and SOX2 | Fig. 1 panel D            |
| Identity                | Quantitative analysis: RT-qPCR     | Comparable expression levels of NANOG, OCT3, and SOX2 compared with H1 | Fig. 1 panel E            |
| Mutation analysis (IF APPLICABLE) | Sequencing            | Tag insertion in one allele, small deletion in the other allele, no apparent off-target effect | Supplementary figure 1    |
| Microbiology and virology | Mycoplasma                       | Mycoplasma test shows negative             | Supplementary figure 3    |
| Differentiation potential | Teratoma formation                | Differentiation to three germ layers confirmed by H&E staining | Fig. 1 panel H            |
| 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                       |

single-molecule super-resolution microscopy in live cell (Chen et al., 2015), here we genetically tagged ATOX1 with fluorescence protein in the H1 stem cell to generate the mCherry-ATOX1 knocked-in (H1_mCh-ATOX1, KI) clone.

We genetically modified H1 human embryonic stem cells (hESCs) by CRISPR-Cas9 mediated homologous recombination (Fig. 1A). H1 hESCs were co-transfected with the single-guide RNAs (sgRNAs)-Cas9 containing plasmids together with corresponding recombination donor plasmids. After puromycin selection, the emerged hESC clones were picked and screened for mCherry insertion by Southern blotting (Suppl. Fig. 1) and DNA sequencing. We obtained a heterozygous mCherry-null allele. G-band karyotyping demonstrated expression of pluripotency transcription factors NANOG, OCT3/4, and SOX2 in KI clone (Fig. 1C). This KI clone is free of mycoplasma contamination tested by PCR-based method (Table 1). Immunofluorescence staining demonstrated expression of pluripotency transcription factors NANOG, OCT3/4, and SOX2 in KI clone (Fig. 1D). We further quantified the transcription of all three pluripotency genes, ATOX1, and mCherry by qRT-PCR (Fig. 1E). Compared with the parental H1 hESCs, KI cells showed comparable expression of all three pluripotency genes (NANOG, 97 ± 3%; OCT4, 87 ± 6%; and SOX2, 107 ± 2%), but the level of ATOX1 transcript in KI clone was reduced (26 ± 1%). Despite the reduced transcript level, immunoblotting results against ATOX1 showed increased mCherry-ATOX1 protein level in the parental KI hESCs compared to the endogenous ATOX1 in the parental H1 lysates (Fig. 1F), probably due to improved protein stability. The un-tagged ATOX1, expected to be endogenous ATOX1 expressed from mCherry-null allele, was barely detectable in the KI hESC lysate, suggesting that endogenous ATOX1 was effectively knocked out in the mCherry-null allele. G-band karyotype analysis confirmed the normal karyotype of KI hESCs (Fig. 1G). Histological staining of KI hESC-derived teratoma showed characteristics of epithelium (endoderm), cartilage (mesoderm), and melanin (ectoderm), confirming the differentiation capability of KI hESCs to three germ layers (Fig. 1H). The short tandem repeat (STR) profile also confirmed that the KI cell still maintains its identity to the parental H1 hESCs (data available upon request). The characterization of KI hESCs is summarized in Table 1.

In summary, the fluorescence KI hESC clone is pluripotent and possesses normal karyotypes. With fluorescence protein mCherry directly tagged on ATOX1 whose expression is still controlled by endogenous regulatory machinery, KI hESCs enable imaging-based approaches to study the cell-type-specific function of ATOX1.

3. Materials and methods

3.1. Cell culture

H1 hESCs (NIH Registration Number: 043, WiCell, passage 28–30) were maintained in mTeSR™-1/Matriigel™ feeder-free system at 37 °C with 5% CO2 until cells reach 80% confluent. Cells were genetically engineered via CRISPR/Cas9 mediated homologous recombination by co-electrotransfecting sgRNA-Cas9 plasmids and homology-direct recombination (HDR) donor plasmid. Transfected cells were plated on puromycin-resistant MEF feeders overnight followed by puromycin selection for four days. Clones were picked and seeded in plates under feeder-free culture system again then passaged for clonal expansion and characterization.

3.2. CRISPR plasmid design

We adopted the protocol from Ran et al. (Ran et al., 2013) and designed the sgRNAs using web tool from Benchling. The sgRNAs targeting to the upstream of ATOX1 coding region (Table 2) were cloned into the pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid (Addgene). A 3 kb genome region covering ATOX1 exon 2 (NC_000005.10, 151,757,164..151,760,261) were PCR amplified from H1 genome and cloned into pMiniT2 plasmid (NEB) as HDR arm. The mCherry sequence was PCR amplified and assembled into the donor plasmid by seamless cloning (NEB).

3.3. PCR, sequencing, and str

Genomic DNA was isolated from ~2 x 106 cells by using MasterPure™ Complete DNA and RNA Purification Kit (Epicentre). PCR was performed using primers (Table 2) and AccuStart™ II GelTrack PCR SuperMix (QuantaBio). PCR protocol: 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 60 °C for 10 s, and 68 °C for 3.5 min; and 72 °C for 5 min. Sanger sequencing was performed by Eton Bioscience. STR DNA fingerprinting was done by the CCGS-funded Characterized Cell Line Core Facility (The University of Texas M.D. Anderson Cancer Center). The STRs at 8 loci were assessed.

3.4. qRT-PCR

Total RNA was isolated the same way as DNA. Two microgram of total RNA were converted to cDNA by using All-in-One RT MasterMix
Table 2
Reagents details.

| Antibodies used for immunochemistry/immunoblotting | Antibody | Dilution | Company Cat # and RRID |
|---------------------------------------------------|----------|----------|------------------------|
| Pluripotency Markers                               | Goat anti-NANOG | 1:500 (ICC) | R&D Systems Cat# AF1997 RRID: AB_355,097 |
| Mouse anti-OCT3/4                                 | 1:250 (ICC) | Santa Cruz Biotechnology Cat# sc-9081, RRID:AB_2,167,703 |
| Goati anti-SOX2                                   | 1:250 (ICC) | Santa Cruz Biotechnology Cat# sc-17,320, RRID:AB_2,286,684 |
| Characterization Markers                          | Rabbit anti-mCherry | 1:500 (ICC) | Abcam Cat# ab67453, RRID:AB_D,571,870 |
|                                                    | Rabbit anti-A TOX1 | 1:1000 (IB) | Abcam Cat# ab154179 RRID: |
| Secondary antibodies                              | Mouse anti- β Actin | 1:2000 (IB) | Sigma-Aldrich Cat# A5441, RRID:AB_476,744 |
| Donkey anti-Goat IgG (Alexa Fluor488 conjugate)   | 1:1000 (ICC) | Jackson Immunoresearch Labs Cat# 705-545-003, RRID:AB_2,340,428 |
| Donkey anti-Mouse IgG (Alexa Fluor594 conjugate)  | 1:1000 (ICC) | Thermo Fisher Scientific Cat# A-21,202, RRID:AB_141,607 |
| Donkey anti-Rabbit IgG (Alexa Fluor594 conjugate) | 1:1000 (ICC) | Thermo Fisher Scientific Cat# A-21,207, RRID:AB_141,637 |
| Peroxidase-AffiniPure Goat Anti-Mouse IgG         | 1:10,000 (IB) | Jackson Immunoresearch Labs Cat# 115-035-003, RRID:AB_10,015,289 |
| Peroxidase-AffiniPure Goat Anti-Rabbit IgG        | 1:10,000 (IB) | Jackson Immunoresearch Labs Cat# 111-035-045, RRID:AB_2,537,938 |

| Primers                                          | Target | Forward/Reverse primer (5’–3’) |
|-------------------------------------------------|--------|-------------------------------|
| CRISPR Plasmid 1 (sgRNA)                        | ATOX1  | CACCCAGGGCTCTCTGGATTCGGGA/ AAAAACTCGGAATTCGAGAGGGGCT |
| CRISPR Plasmid 2 (sgRNA)                        | ATOX1  | CACCCAGGGCTCTCTGGATTCGGGA/ AAAAACTCGGAATTCGAGAGGGGCT |
| HDR Genome                                      | ATOX1 inton 1–2 | TTCCTCCAGGTTATCTGTTGTA/ AACTTCTTCTGTACGTTCTGTGTT |
| Site direct mutagenesis                         | PAM of ATOX1 sgRNA 1 | GCGGAGAGCTCTGCGGTAAC/ TGCCGAGCTCAGTGGG |
| Site direct mutagenesis                         | PAM of ATOX1 sgRNA 2 | GCGGAGAGCTCTGCGGTAAC/ TGCCGAGCTCAGTGGG |
| Donor Plasmid Assembly                          | ATOX1-mCherry | GCCGAGGGATATCTCTGGAG/ GGCTCGCTGGCAACAGAAGAGCAGAG |
| Donor Plasmid Assembly                          | mCherry | GCCGAGGGATATCTCTGGAG/ GGCTCGCTGGCAACAGAAGAGCAGAG |
| Pluripotency Markers (pCR)                      | NANO6 | TTTTGGGGCTGAAGAATACCT/ AGGCGTCTACGGAATACAGCAG |
| Pluripotency Markers (pCR)                      | OCT4  | AAATTCGCTGATCTTGCCAGGTT/ TGAGACTCTCTACTGGCAACAG |
| Pluripotency Markers (pCR)                      | SOX2  | AGAGAGAGAGAGGCAAGAGAGAG/ AGAGAGAGAGGCAACAGG |
| Target mutation analysis (pCR)                  | ATOX1  | CATGCGGAGGCGGAGA/ TCTCGAGGTCAGGGGAGAGGAGAG |
| Target mutation analysis (pCR)                  | mCherry | CCGGCTAAGCTGTGGGTG/ CTTCGTCGTTGCAAGG |
| House-Keeping Genes (pCR)                       | GAPDH | CAACACTCCTACCTGGAAC/ ACCCTTCTGCTTGTAAGG |
| Genotyping                                     | ATOX1 exon 2 | GGGCCCTTTTCAGTCAAGAAT/ GTGACGGCTTTCTCCG |

| Detection Kit (Applied Biological Materials Inc.) | qPCR was performed using primers (Table 2) following the protocol of IQ SYBR Green Supermix (BioRad). Results were calculated using the ΔΔCT method and normalized to GAPDH expression. |

3.5. Immunoblotting and immunofluorescence staining

An equal amount of total protein from cell lysate was resolved in a SDS-PAGE for immunoblotting analysis. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS for 15 min, blocked and incubated with primary antibodies (Table 2) overnight at 4 °C, incubated with secondary antibodies, and counterstained with DAPI.

3.6. Karyotyping

The cultures were harvested and G-banded using standard protocols. Clonal chromosomal changes identified by G-banding were described according to an International System for Human Cytogenetic Nomenclature ISCN (McGowan-Jordan & An, n.d.). Twenty metaphase chromosome spreads at 400 band resolution were analyzed.

3.7. Teratoma assay

Cells grown under log-phase were dissociated and resuspended in 50% Matrigel/DMEM-F12. Immunodeficient NOD SCID mice (Charles River Laboratories) were injected subcutaneously with 2 × 107 cells in each flank and observed for palpable teratoma formation. After six weeks, teratomas were excised then fixed in 10% formalin (Sigma). Histology was analyzed using H&E staining.

3.8. Mycoplasma testing

Mycoplasma detection was performed using PCR Mycoplasma (Applied Biological Materials Inc). qPCR was performed using primers (Table 2) following the protocol of IQ SYBR Green Supermix (BioRad). Results were calculated using the ΔΔCT method and normalized to GAPDH expression.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.106131.

References

Celauro, E., Mukai, A., Fierro-Gonzalez, J.C., Wittung-Stafshede, P., 2017. Copper chaperone ATOX1 regulates pluripotency factor OCT4 in preimplantation mouse embryos. Biochem Biophys Res Commun 491, 147–153.

Chen, T.-Y., Santiago, A.G., Jung, W., Krzeminski, L., Yang, F., Martell, D.J., Helmann, J.D., Chen, P., 2015. Concentration- and chromosome-organization-dependent regulator unbinding from DNA for transcription regulation in living cells. Nat Commun 6, 6848–6852.

Dzebo, Matson, M., Blockhuys, S., Valenzuela, S., Celauro, E., Mukaj, A., Fierro-Gonzalez, J.C., Wittung-Stafshede, P., 2017. Copper chaperone atox1 interacts with cell cycle proteins. Proc Natl Acad Sci U S A 98, 6848–6852.

Dzebo, Matson, M., Blockhuys, S., Valenzuela, S., Celauro, E., Esbjorner, E.K., Wittung-Stafshede, P., 2018. Copper chaperone ATOX1 regulates pluripotency factor OCT4 in preimplantation mouse embryos. Biochem Biophys Res Commun 491, 147–153.

J.-D., Chen, P., 2015. Concentration- and chromosome-organization-dependent regulator unbinding from DNA for transcription regulation in living cells. Nat Commun 6, 6848–6852.

Hamza, I., Faisit, A., Phokhara, J., Chen, J., Gruss, P., Gitlin, J.D., 2001. The metallochaperone atox1 plays a critical role in perinatal copper homeostasis. Proc Natl Acad Sci U S A 98, 6848–6852.

Dzebo, Matson, M., Blockhuys, S., Valenzuela, S., Celauro, E., Esbjorner, E.K., Wittung-Stafshede, P., 2018. Copper chaperone atox1 interacts with cell cycle proteins. Proc Natl Acad Sci U S A 98, 6848–6852.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., Zhang, F., 2013. Genome engineering using the CRISPR-Cas9 system. Nat Protoc 8, 2281–2308.