Applied Microbiology and Biotechnology (2019) 103:2171–2180
https://doi.org/10.1007/s00253-019-09615-3

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Establishment and characterization of an immortalized renal cell line of the Chinese tree shrew (*Tupaia belangeri chinesis*)

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Received: 31 October 2018 / Revised: 27 December 2018 / Accepted: 29 December 2018 / Published online: 12 January 2019

Abstract

The Chinese tree shrew holds a great potential as a viable animal model in biomedical research, especially for infectious diseases and neuropsychiatric disorders. A thorough understanding of the innate immunity, which represents the first line that defends the host against viral infection, of the Chinese tree shrew, is needed. However, the progress is hindered by the lack of a proper cell line for research usage. In this study, we established a cell line that is applicable to the study of tree shrew innate immune responses against viral infections. The Chinese tree shrew primary renal cells (TSPRCs) were immortalized by simian virus 40 large T antigen (SV40LT) transduction, and the immortalized cells were termed TSR6 (tree shrew renal cell #6). TSR6 showed a similar morphology to TSPRCs and expressed the epithelial cell-specific marker cytokeratin 18 (KRT18). In addition, TSR6 could be transfected by transfection reagent and was suitable for CRISPR/Cas9-mediated gene editing. Infection of Newcastle disease virus (NDV) or herpes simplex virus 1 (HSV-1) in TSR6 induced the mRNA expression of tree shrew interferon-β (*tIFNB1*) and myxovirus resistance protein 1 (*tMx1*) in a dose- and time-dependent manner. Collectively, we successfully established a tree shrew renal cell line and demonstrated that this cell line was suitable for the study of the innate immune response to viral infections.

Keywords Tree shrew · Cell line · Viral infection · Innate immune response

Introduction

The Chinese tree shrew is a squirrel-like and rat-sized mammal that widely distributes in Southeast Asia, South, and Southwest China (Hubrecht et al. 2010; Xu et al. 2013a; Yao 2017; Zheng et al. 2014). It shares a close relationship to the primate and has many advantages as an experimental animal for biomedical researches, such as a small body size (100–150 g), a low-cost of maintenance, a short reproductive cycle (~6 weeks), and life span (6–8 years) (Fan et al. 2013; Tsukiyama-Kohara and Kohara 2014; Xiao et al. 2017; Xu et al. 2012; Yao 2017).

To this day, increasing reports have showed that the Chinese tree shrew could be used as an experimental animal for the study of viral infectious diseases (Li et al. 2018; Tsukiyama-Kohara and Kohara 2014; Xiao et al. 2017; Xu et al. 2013b; Yao 2017). Inoculated newborn tree shrews with the sera from hepatitis B virus (HBV)-infected patients or tree shrews led to hepatic histopathological changes in these animals that were resembled to that of HBV-infected humans (Ruan et al. 2013). Hepatitis C virus (HCV)-infected tree shrews showed mild hepatitis and intermittent viremia during the acute phase of infection (Amako et al. 2010). The pathologic changes of chronic hepatitis were observed, and the infectious virus particles were produced in the livers of infected tree shrews (Amako et al. 2010). Besides HBV and HCV, tree shrews were also susceptible to other human viruses (Li et al. 2018; Xiao et al. 2017). For instance, herpes simplex virus 1 (HSV-1) and HSV-2 could latently infect tree

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shrew peripheral nervous system sensory neurons, and the viral reactivation from latency leads to recurring cold sores in tree shrew (Li et al. 2016). Experimentally, Coxsackie virus A16-infected tree shrews showed increased body temperature and pathological changes in central nervous system and other organs (Li et al. 2014). The human H1N1 influenza virus-infected tree shrews displayed mild or moderate systemic and respiratory symptoms and pathological changes in respiratory tracts (Yang et al. 2013).

Despite the fact that tree shrew is closely related to the primate and can mimic the pathology of human virus infections, the tree shrew had its unique genetic features in the innate immune system that were revealed by the analysis of the Chinese tree shrew genome (Fan et al. 2013). For instance, the most important cytoplasmic pattern-recognition receptors (PRRs) retinoic acid-inducible gene I (RIG-I) was absent in the tree shrew, which leads to a functional replacement with MDA5 (Xu et al. 2016). Many genes that are involved in the innate immune response were under positive selection in the Chinese tree shrew, such as toll-like receptors 8 (*TLR8*) and *TLR9* (Yu et al. 2016). To use the tree shrew as a mature and stable experimental animal in the viral infectious disease research, comprehensive elucidation of the innate immune is a prerequisite. However, the absence of a suitable cell line impedes this progress.

In this study, we immortalized and established a tree shrew renal cell line that was designated TSR6 (tree shrew renal cell #6). The TSR6 was identified as epithelial lineages based on the expression pattern of cytokeratin 18 (KRT18) and vimentin (VIM), which were lineage-specific markers for epithelial and fibroblasts cells, respectively. TSR6 could be transfected and was suitable for the CRISPR/Cas9-mediated gene editing. We provided further evidence to show that the TSR6 could be used for the study of tree shrew innate immune response to viral infections.

Materials and methods

Experimental animals

The Chinese tree shrews were purchased from the experimental animal core facility of the Kunming Institute of Zoology, Chinese Academy of Sciences. All efforts were made to minimize the suffering of animals.

Isolation and culture of the tree shrew primary renal cells

The tree shrew was lethally anesthetized by pentobarbital and the kidney was quickly harvested. The isolation of tree shrew renal cells was described in our previous study (Xu et al. 2016). Briefly, the kidney was minced into small pieces and digested with 1 mg/mL DNase I (Sigma, AMPD1-1KT) and 5 mg/mL collagenase type IV (Invitrogen, 17104019) solution for 45 min in a 37 °C water bath. After washed three times with cold phosphate-buffered saline (PBS; biological industries, 0021517), the tree shrew primary renal cells (TSPRCs) were resuspended and cultured at a density of 2×10^5 cells/mL in high-glucose Dulbecco's modified eagle medium (DMEM; Gibco-BRL, 11965-092) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, 10099-141) and 1 × penicillin/streptomycin (Gibco-BRL, 10378016) at 37 °C in 5% CO_2 until confluent.

Cells and lentiviral transduction

HEK293T cells were supplied by the Kunming Cell Bank, Kunming Institute of Zoology, Chinese Academy of Sciences. HEK293 cells were cultured in high-glucose Dulbecco's modified eagle medium (DMEM; Gibco-BRL, 11965–092) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, 10099–141) and 1 × penicillin/streptomycin (Gibco-BRL, 10378016) at 37 °C in 5% CO_2.

The cells were seeded into six-well plates at a density of 4×10^5 cells/well and co-transfected with 0.4 μg of pMD2.G (Addgene, 12259), 0.8 μg of psPAX2 (Addgene, 12260), and 1.3 μg of pLVX-SV40LT-puro plasmid (a kind gift from Dr. Ping Zheng, Kunming Institute of Zoology). The viral supernatants were harvested and filtered with 0.45 μm filters at 48 h post-transfection. For the lentiviral infection, TSPRCs were seeded in six-well plates at a density of 4×10^5 cells/well. After 12 h, the culture medium was replaced by infection mixture [500 μL cultural medium with 1 μg polybrene (Solarbio, H8761) and 500 μL viral supernatants]. Puromycin (1 μg/mL; Solarbio, P8230) was added to the culture medium at 48 h post-infection, and the puromycin-resistant cells were pooled and expanded.

Cell growth kinetics

The TSR6 cells were seeded in 12-well plates at a density of 1×10^5 cells/well for growth. Cells in three wells were harvested by trypsinization (0.25% Trypsin-EDTA; Gibco, 1806021) for five consecutive days, the cell numbers were counted by Countstar BioTech (Countstar, China). The growth curve of cells was plotted.

Viral infections

HSV-1 was obtained from Dr. Jumin Zhou's laboratory at the Kunming Institute of Zoology, Chinese Academy of Sciences. NDV was obtained from the China Institute of Veterinary Drug Control. All the viruses were propagated and amplified

following the previously described procedures (Xu et al. 2015, 2016, 2019).

The TSR6 cells were seeded in 24-well plates at a density of 2×10^5 cells/well and infected with NDV or HSV-1 at a multiplicity of infections (MOI) of 1, 2, and 5 for 1 h, respectively. Cells were then washed by PBS twice, and fresh growth medium was added. The cells were harvested at 6 h and 12 h post-infection.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using RNAsimple Total RNA Kit (TIANGEN, DP419) according to the manufacturer's instruction. Complementary DNA was synthesized by using random primer and M-MLV reverse transcriptase (Promega, M1701). The quantitative real-time PCR was performed using SYBR green Premix Ex Taq II (TaKaRa, RR820L) supplemented with gene-specific primers on a CFX Connect Real-Time System (Bio-Rad, USA) as described previously (Xu et al. 2016). The primers for *tIFNB1* and *tMx1* were described in our previous studies (Xu et al. 2015; Yu et al. 2014, 2016). The primers for tree shrew *KRT18* (*tKRT18*: 5′-GAGTACCAGGAGCTCATGAATGT-3′/5′-TGTTCTGCATCCCAGATTCCA-3′) and for *tVIM* (5′-GCAGGATGAGATTCAGAAC-3′/5′-CTTAACATTGAGCAGGTCTT-3′) were newly designed based on the full-length mRNA sequences of *tKRT18* and *tVIM* from the tree shrew database: treeshrewDB (http://www.treeshrewdb.org) (Fan et al. 2014).

DNA transfection

TSR6 cells were seeded in six-well plates at a density of 5×10^5 cells/well, and the cell culture medium was replaced by Opti-MEM (Gibco-BRL, 31985-070) before transfection. For transfection, 2 μg of pEGFP-N2 (Clontech, #6081-1) was diluted in 200 μL of Opti-MEM, then 4 μL of X-tremeGENE HP (Roche, 06366546001) was added to form the transfection reagent-plasmid DNA complex. After incubation for 15 min at room temperature, the transfection mixture was added to cells in a dropwise manner. At 5 h post-transfection, the Opti-MEM was replaced by growth medium. The GFP expression was visualized at 24 h post-transfection under a fluorescence microscope, and cells were collected at 48 post-transfection for flow cytometry analysis. We also tested the transfection by Lipofectamine 3000 (Thermo Fisher, L300015). In brief, 3.75 μL of Lipofectamine 3000, 2.5 μg of plasmid DNA, and 5 μL of P3000 (Thermo Fisher, L300015) were diluted in 125 μL Opti-MEM to form Lipofectamine 3000-plasmid DNA-P3000 complex. The procedures for transfection, detection of GFP expression, and flow cytometry analysis were same as above.

Overexpression and knockdown of tree shrew melanoma differentiation factor 5 (tMDA5)

Flag-tagged tMDA5 plasmid (pCMV-tMDA5-3tag) and siRNA targeted to *tMDA5* had been described in our previous study (Xu et al. 2016). We used the Lipofectamine 3000 (Thermo Fisher, L300015) for the transfection, as it had a higher transfection efficiency compared to X-tremeGENE HP (Roche, 06366546001). The TSR6 cells were seeded in six-well plates (5×10^5 cells/well) and were transfected with pCMV-tMDA5-3tag as described above. siRNA transfection (100 nM/well) was performed following the protocol as described for overexpression vector but without P3000 reagent. Cells were counted at 48 h post-transfection and collected for quantitative real-time PCR and Western blot analyses.

CRISPR/Cas9-mediated knockout of tMDA5 in TSR6

The sgRNA targeting sequence for the *tMDA5* gene was designed by using the CRISPR Design Tool (http://bioinfogp.cnb.csic.es/tools/breakingcas/index.php) (Oliveros et al. 2016). The sgRNA targeting sequence pair (tMDA5-sgRNA-F: 5′-TAGACAAGCAGTTCCGCTAT-3′/tMDA5-sgRNA-R: 5′-ATAGCGGAACTGCTTGTCTA-3′) were cloned into the pX330-T7 vector (a kind gift from Dr. Ping Zheng, Kunming Institute of Zoology) expressing mCherry following the previous report (Ran et al. 2013). The TSR6 cells were transfected by using Lipofectamine 3000. Cells expressed mCherry were sorted by flow cytometry and cultured for 48 h, then single cells were manually picked with a mouth pipette for expansion after culture for around 3 weeks.

Genomic DNA was extracted using AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, 26817KC1). The gene region spanning the sgRNA targeting site was amplified by primer pair (tMDA5-sgRNA-2Fc: 5′-GCTAGAGGACCCTGCACCAG-3′/tMDA5-sgRNA-2Rc: 5′-CAGGCAGAAAGGTCAGGTAG-3′). The PCR products were first sequenced using the PCR primer tMDA5-sgRNA-2Fc to screen which sample contains potential mutation(s). Subsequently, the PCR products containing potential mutation(s) were cloned into T-Vector pMD19 (TaKaRa, 3271). About 10 clones were randomly selected for sequencing for each transformation. The sequence was analyzed by using the DNASTAR Lasergene 7.1 (DNAS Inc., Madison, WI, USA). Cells containing mutation(s) were further analyzed by Western blot to confirm the successful knockout of *tMDA5*.

Western blot and immunofluorescence

Cells were harvested by trypsinization and washed three times with cold PBS. The collected cells were lysed on ice in RIPA

lysis buffer (Beyotime, P0013), followed by centrifugation at 12,000×*g* at 4 °C for 10 min to remove cell debris. The protein concentration was determined using the BCA protein assay kit (Beyotime, P0012) following the manufacturer's instructions. A total of 20 μg protein was separated by electrophoresis on a 12% (vol/vol) SDS-polyacrylamide gel and transferred to PVDF membranes (Bio-Rad, #1620177). The membranes were blocked with 5% (wt/vol) bovine serum albumin (BSA; Amresco, 0332) in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) (Cell Signaling Technology, #9997) at room temperature for 2 h. The membranes were then incubated with primary antibodies against the KRT18 antibody (EnoGene, E1A0191), the VIM antibody (EnoGene, E1A7013), and the MDA5 antibody (Merck Millipore; ABF210), respectively, overnight at 4 °C. After three washes with TBST, the membranes were incubated for 1 h with peroxidase-conjugated anti-rabbit (KPL, 074–1506) IgG (1:10,000) at the room temperature. The epitope was visualized by using an ECL Western blot detection kit (Millipore, WBKLS0500).

For immunofluorescence assay, cells were seeded in chamber slide (Thermo, 154526) for 12 h and washed three times with PBS before being fixed by 4% paraformaldehyde. After having been permeated with 0.2% Triton X-100 for 15 min and three washes (each 5 min), cells were incubated with the primary antibodies against KRT18 and VIM, respectively, overnight at 4 °C. After another round of three washes with PBS, cells were incubated with the secondary antibody (Invitrogen, A-21207) for 1 h. Nuclei were stained by DAPI (Roche, 10236276001). Intact cells were imaged by using an Olympus FluoView™ 1000 confocal microscope (Olympus).

Statistical analysis

Statistical significance was determined by using the unpaired Student's *t* test with Prism software (GraphPad). Significant values were indicated as $*p < 0.05$ and $**p < 0.01$. Results were represented as mean ± standard error of mean (SEM). Data shown were representatives of at least three independent experiments, which showing similar results.

Results

Establishing the TSR6 cell line

To establish a stable tree shrew renal cell line, TSPRCs were transducted with the lentivirus containing the SV40LT and were selected by puromycin treatment. While the TSPRCs died out at passage 3, the stably transducted cells continued to replicate to passage 40 (Fig. 1a). Single cells were cultured for monoclonal cell formation, and we finally obtained a monoclonal cell line, which was termed as tree shrew renal cell #6 (TSR6). Both the TSPRCs and TSR6 exhibited similar morphology; no obvious morphological changes were observed during prolonged passage in culture (Fig. 1a). The hallmark of immortalized cells is that it could continuously proliferate. We determined the growth kinetics of TSR6. As shown in Fig. 1b, TSR6 began to enter a fast growing period at 48 h after adherence to culture plate. We cultured the TSR6 cells up to 100 passages, and we found no essential difference in growth rate between different

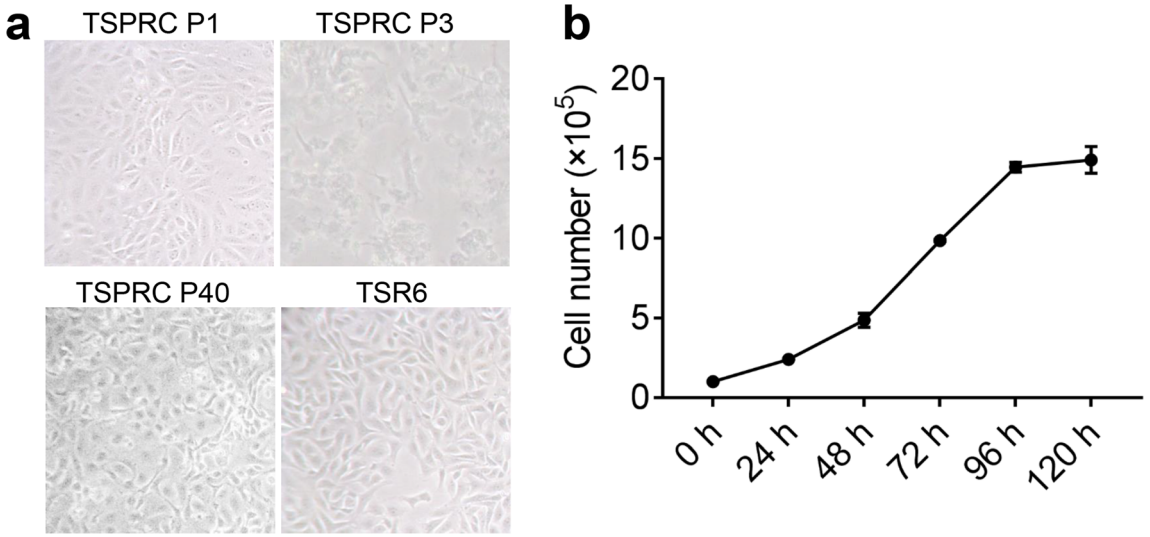

Fig. 1 Morphological characterization of the Chinese tree shrew primary renal cells (TSPRCs) and cell line TSR6. **a** Cellular morphology of TSPRCs at passages 1 and 3 (TSPRC P1 and TSPRC P3), SV40LT transduced TSPRCs at passage 40 (TSPRC P40), and the TSR6 cells. All cells were magnified by 100 times. **b** The growth curve of the TSR6 cells. Cells were seeded in 12-well plates at a density of 1×10^5 cells/well. We counted the cell numbers each day for five consecutive days

generations. Taken together, these results suggested that the TSR6 was a stable cell line. This cell line has been deposited in the Kunming Cell Bank, Kunming Institute of Zoology, Chinese Academy of Sciences (KCB No: KCB2018047YJ).

Lineage of the TSR6 cells

The lineage of the TSR6 was determined by detecting the KRT18 and VIM expressions, which are markers for epithelial and fibroblast cells, respectively (Nelson 1983; Wang et al. 2014). Immunofluorescence analysis showed that both the tKRT18 and tVIM were expressed in TSR6 (Fig. 2a). Compared to TSPRCs, the TSR6 had a significantly increased mRNA level of *tKRT18* and a significantly decreased level of *tVIM*. In accord with mRNA expression, the protein level of tKRT18 was elevated in TSR6, whereas tVIM protein expression showed no difference between TSR6 and TSPRCs (Fig. 2b, c). Although VIM is a marker of fibroblast cell, it has also been detected in some specific epithelial cells, such as mammary epithelial cells (Mork et al. 1990). In contrast, KRT18 is exclusively expressed in epithelial cells (Nelson 1983). It was therefore reliable to conclude that the TSR6 is an epithelial lineage cell line.

TSR6 can be transfected by transfection reagents

Ectopic expression and siRNA-mediated knockdown are the most common experiments for immunology research. We firstly investigated the expression level of EGFP in TSR6 by using commercial transfection reagents. Both X-tremeGENE HP and Lipofectamine 3000 could successfully deliver the EGFP vector into TSR6 and produce the EGFP protein (Fig. 3a). All the transfections had a mild effect on cell viability (Fig. 3b), but the Lipofectamine 3000 exhibited a higher transfection efficiency than X-tremeGENE HP (Fig. 3a). We further performed overexpression and knockdown assays for tMDA5, which were carried out on TSPRCs in our previous study (Xu et al. 2016), by using Lipofectamine 3000 for TSR6 cells. Evidently, siRNA transfection knocked down the expression of tMDA5 at both mRNA and protein levels (Fig. 3c, d), whereas overexpression of tMDA5 generated the corresponded protein in TSR6 cells (Fig. 3e). These results indicated that the TSR6 cells could

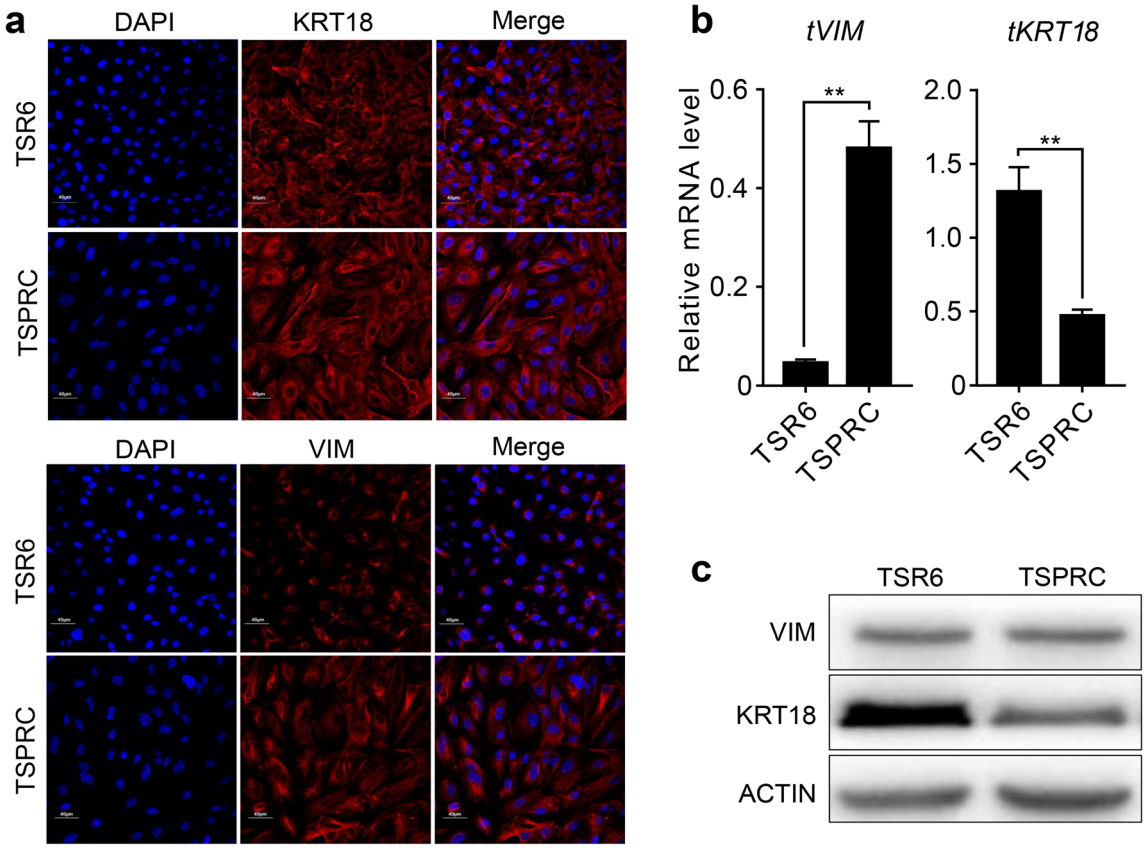

Fig. 2 Expression of tKRT18 and tVIM in the Chinese tree shrew primary renal cells (TSPRCs) and cell line TSR6. **a** TSPRCs and TSR6 were stained with KRT18 and VIM antibodies (red), and cell nuclei were stained by DAPI (blue). The mRNA (**b**) and protein (**c**) levels of KRT18 and VIM were analyzed by quantitative real-time PCR and Western blot assays, respectively

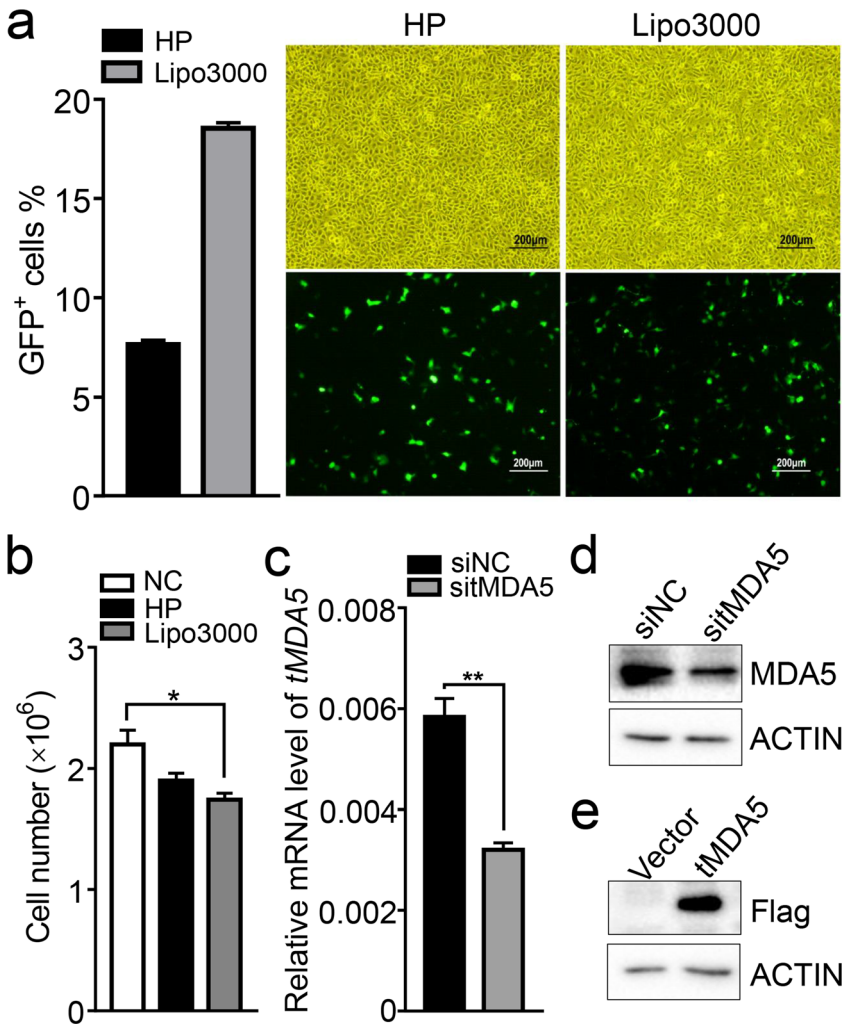

Fig. 3 Transfection efficiency of the TSR6 cell line. **a** TSR6 cells were transfected with pEGFP-N2 by X-tremeGENE HP (HP) or Lipofectamine 3000 (Lipo3000) for 24 h, then checked for EGFP expression by a fluorescence microscope (× 100 magnification). The ratio of EGFP expressing cells was estimated by flow cytometry at 48 h post-transfection. **b** Quantification of cell viability after transfection. The non-transfected (NC) and transfected (HP and Lipo3000) TSR6 cells were cultured for 48 h before counting the cell number. The expression levels of *tMDA5* mRNA (**c**) and protein (**d**) in TSR6 cells transfected with the negative control siRNA (siNC) and sitMDA5. Transfected cells were harvested at 48 h post-transfection and were analyzed by quantitative real-time PCR and Western blot. **e** Overexpression of tMDA5 in TSR6 cells. Cells were transfected with empty vector (Vector) and pCMV-tMDA5-3tag (tMDA5), respectively. Expression of tMDA5 protein was analyzed by Western blot. The tree shrew housekeeping gene *β-actin* was used as the control for normalization

be effectively transfected and used to perform the ectopic expression and knockdown experiments.

TSR6 was suitable for CRISPR/Cas9-mediated gene editing

The CRISPR/Cas9 technology has been widely used to facilitate efficient genome engineering in eukaryotic cells by simply specifying a 20-nt targeting sequence within its guide RNA (Ran et al. 2013), and this technology has opened a broad avenue for molecular biology (Chen et al. 2016; Jin and Li 2016; Luo et al. 2016; Ma et al. 2018). We tested whether CRISPR/Cas9 can work in TSR6. A sgRNA targeting sequence to disrupt the gene encoding tMDA5 was annealed and ligated into the pX330-T7 vector expressing mCherry. The targeting plasmid expressing sgRNA and Cas9 proteins was delivered into TSR6 by transfection. About 48 h post-transfection, cells expressing mCherry were sorted by flow cytometry. Single cells were picked up for growing into individual colonies. A disrupt mutation c.21_22insA was observed in the exon 1 of *tMDA5* in two single cell colonies KO9 and KO10 (Fig. 4a). This single base insert leads to knockout of tMDA5 protein (Fig. 4b). Thus, the CRISPR/Cas9-mediated-gene editing could work in TSR6.

The TSR6 cell line has an innate immune response to viral infection

After sensing viral invasion, interferons (IFNs) are produced to induce the expression of interferon-stimulated genes (ISGs) to establish an antiviral state (Borden et al. 2007; Schneider et al. 2014). Currently, the mRNA expressions of IFNs and ISGs are major markers to indicate innate immunity evocation as the IFNs and ISGs mRNA are not detectable during a resting state (Borden et al. 2007; Schneider et al. 2014). To assess whether viral infection could induce the innate immune response in TSR6, the mRNA expression levels of *tIFNB1* and *tMx1*, which belong to IFNs and ISGs respectively, were determined in TSR6 after challenge with RNA virus NDV and DNA virus HSV-1. In general, NDV infection induced the *tIFNB1* expression in a dose- and time-dependent manner (Fig. 5a). Specifically, higher titer of NDV induced a higher mRNA level

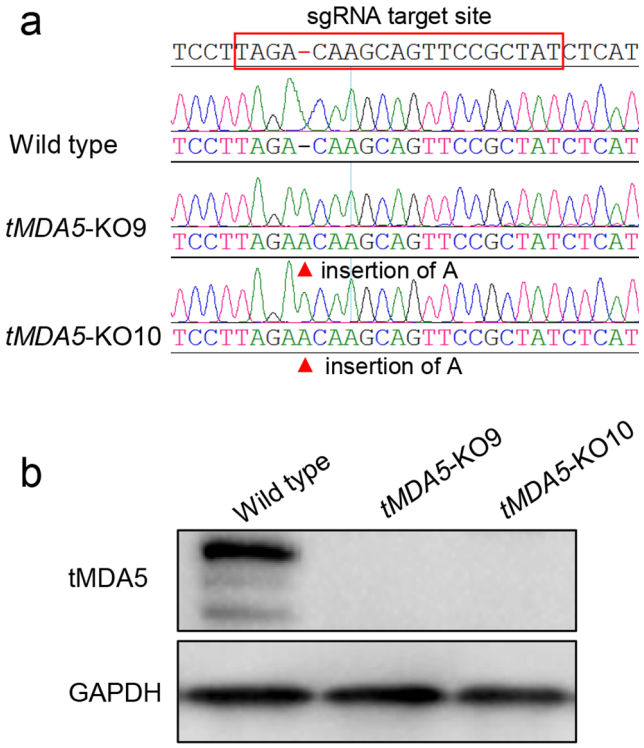

Fig. 4 Successful knockout of the *tMDA5* gene in TSR6 cells by using the CRISPR/Cas9 technology. **a** Sequencing chromatographs showing the introduced mutations in the *tMDA5* gene in two lines of TSR6 cells with gene editing (tMDA5-KO9 and tMDA5-KO10) and the unedited TSR6 cells (wild type), and **b** Western blot for the tMDA5 protein in these cells. The GAPDH was used as the loading control in the Western blot

of *tIFNB1*. At 1 MOI of NDV, the mRNA expression level of *tIFNB1* was only slightly but significantly induced, while at 2 MOI and higher titers, the *tIFNB1* mRNA was induced at a significantly higher level compared to that of 1 MOI of NDV. In cells infected with the same concentration of NDV, the *tIFNB1* mRNA expression was significantly increased at 12 h compared to 6 h. The mRNA level of *tMx1* was induced as early as 12 h after NDV infection in a dose-dependent manner (Fig. 5a). Infection with the HSV-1 induced *tIFNB1* mRNA level in a similar way to NDV (Fig. 5b). However, the mRNA level of *tMx1* was significantly induced at 6 h by HSV-1 infection (5 MOI), and higher concentration of HSV-1 dramatically induced the mRNA expression of *tMx1* at 12 h (Fig. 5b). These results indicated that the TSR6 possessed the innate immune response to viral infections.

Discussion

Tree shrew is a promising experimental animal for infectious disease research (Li et al. 2018; Yao 2017; Zheng et al. 2014). At the present, many literatures have described the pathology and physiology of tree shrews with viral infections (Li et al. 2018). In our previous study, we provided a publicly available annotated genome sequence of the Chinese tree shrew and established the tree shrew database (www.treeshrewdb.org) (Fan et al. 2013; Fan et al. 2014). These efforts offered a solid base to elucidate the basic biological properties and to create animal models using this species (Yao 2017). Furthermore, the recent successful genetic manipulation of the tree shrew has opened a new avenue for the wider usage of this animal in biomedical research (Li et al. 2017; Yao 2017). However, the understanding of the innate immunity and host-viral interaction are limited by the lack of a suitable cell line. There was a report for using tree shrew fibroblast cells to study dengue virus infection (Kayesh et al. 2017). In our recent studies to characterize the tree shrew immune genes (Xu et al. 2015, 2016; Yao et al. 2019; Yu et al. 2014, 2016), we used tree shrew primary renal cells, and had to frequently sacrifice the animal for isolating cells. It is necessary to have a cell line that is capable for initial characterization. In this study, we successfully established a tree shrew renal cell line and characterized its cellular responses to viral infections. This cell line could serve as a good resource for studying tree shrew.

Normal mammalian cells have a limited life span in culture; although the spontaneous immortalization could occur, this event had been seldom observed (Maqsood et al. 2013; Ramboer et al. 2014). The SV40LT is frequently used to aid cellular immortalization (Ahuja et al. 2005) via multiple mechanisms (Ramboer et al. 2014). First, it inactivates at least three growth suppressors, pRB, p53, and SEN6 (Ahuja et al. 2005). Second, it induces telomerase activity which is critical in cellular immortalization (Ahuja et al. 2005; Foddis et al. 2002). Besides these effects, the SV40LT possesses other additional activities that could also potentially contribute to cellular immortalization (Ahuja et al. 2005). All these unique roles of SV40LT rendered it as a powerful tool for artificial cellular immortalization. We established the stable tree shrew renal cell line by transducing the SV40LT antigen to TSPRCs. As the TSPRCs were mixtures of many types of cells, cells immortalized from TSPRCs would also contain a heterogeneous cell population. In order to improve cell homogeneity, we picked up single cells to obtain monoclonal immortalized shrew renal cell line TSR6. Morphological analysis showed that both TSPRCs and TSR6 displayed a similar morphology, and TSR6 displayed a good proliferative activity.

The cytokeratin proteins form tonofilaments which are present in almost all vertebrate epithelia (Nelson 1983). Since cytokeratins are usually not detectable in non-epithelial cells including fibroblasts, muscles, and nerves, immunohistochemical staining of cytokeratin facilitates the detection and identification of epithelial cells in tissue section and in culture (Nelson 1983). Cytokeratin 18 immunostaining has traditionally been used for the identification of fully differentiated epithelial cells (Wang et al. 2014). Vimentin is one of the four types of intermediate filaments which play a

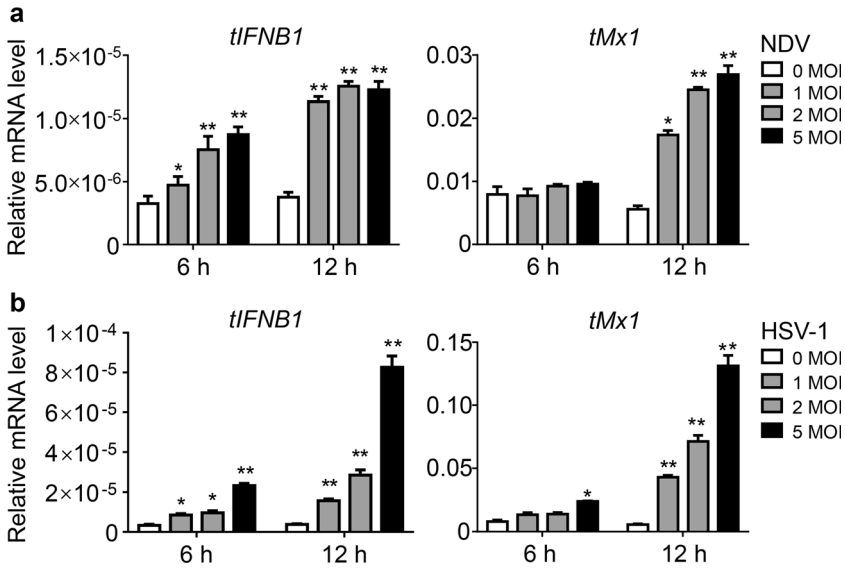

Fig. 5 Upregulation of *tIFNB1* and *tMx1* mRNA levels after **a** NDV and **b** HSV-1 infections in TSR6 cells. Cells were either infected with NDV or HSV-1 at different multiplicity of infections (MOI) or without infection for 6 h and 12 h, respectively. The mRNA levels of *tIFNB1* and *tMx1* were determined by quantitative real-time PCR and were normalized by the tree shrew housekeeping gene *β-actin*

structural and/or tension-bearing role in many cells originating from the mesenchyma, and its expression has been thought to be a fibroblast cell marker (Banerjee et al. 2016; Minin and Moldaver 2008). We detected both cytokeratin 18 and vimentin in TSR6 at the mRNA and protein levels, suggesting that the TSR6 cells were of epithelial-lineage. We further showed that the TSR6 cells could be easily transfected by the commonly used DNA transfection reagents, albeit the transfection efficiency was not very high. Therefore, it would be necessary to further optimize the transfection to achieve a higher efficiency. By way of example, we showed that the CRISPR/Cas9-mediated gene editing and siRNA interference worked in TSR6. Collectively, TSR6 is suitable for clarifying the function of certain gene in vitro by overexpression, knock down, and knockout assays.

The innate immunity is the first line of host defense against pathogens which is initiated by the detection of invading pathogens via PRRs (Akira et al. 2006). After the detection of invading pathogens, PRRs induce the IFNs expression by a series of signal transduction processes, subsequently upregulate the ISGs expression to establish an antiviral state (Akira et al. 2006; McNab et al. 2015; Schneider et al. 2014). NDV is a member of the genus *Rubulavirus* of the subfamily *Paramyxovirinae* (family *Paramyxoviridae*, order *Mononegavirales*) which contains a non-segmented, single-stranded RNA genome of negative polarity (de Leeuw and Peeters 1999). HSV-1 belongs to the *Alphaherpesvirinae* subfamily, and it is a typical double-stranded DNA virus (Su and Zheng 2017). We used both viruses as the representatives of RNA virus and DNA virus for stimulation. Both NDV and HSV-1 induced the *tIFNB1* and *tMx1* mRNA expression in a dose-dependent manner in TSR6. Therefore, the TSR6 possesses an intact innate immune response to viral infections, indicating that this tree shrew cell line is suitable for studying innate immunity upon viral infection.

In summary, we established a stable cell line from the Chinese tree shrew primary renal cells, which retains the morphological features of the primary cells. This cell line could be well transfected by commercial transfection reagents and could be edited by using the CRISPR/Cas9 technology. Furthermore, TSR6 possesses an intact innate immune reaction to viral infections. We believe that this cell line may be helpful for mechanistic studies of tree shrew innate immunity and other in vitro researches.

Funding This study was supported by the National Natural Science Foundation of China (U1402224 to Y-GY), Yunnan Province (2018FB046 to DY), and the Chinese Academy of Sciences (CAS zsys-02 to Y-GY).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal studies with tree shrew were approved by the Institutional Animal Care and Use Committee of Kunming Institute of Zoology, Chinese Academy of Sciences (Approval No: SYDW20110315001).

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