

***Final Draft***  
**of the original manuscript:**

Hildebrand, L.; Seemann, P.; Kurtz, A.; Hecht, J.; Contzen, J.; Gossen, M.;  
Stachelscheid, H.:

**Selective cell targeting and lineage tracing of human induced  
pluripotent stem cells using recombinant avian retroviruses**

In: Cellular and Molecular Life Sciences (2015) Springer

DOI: 10.1007/s00018-015-1957-4

1  
2  
3 **Selective cell targeting and lineage tracing of human induced pluripotent stem cells**  
4  
5 **using recombinant avian retroviruses**  
6

7  
8 Laura Hildebrand<sup>1,2</sup>, Petra Seemann<sup>1,2</sup>, Andreas Kurtz<sup>1,2,4</sup>, Jochen Hecht<sup>1,2,6</sup>,  
9  
10 Jörg Contzen<sup>2,3</sup>, Manfred Gossen<sup>2,3</sup>, Harald Stachelscheid<sup>1,2,5</sup>  
11

- 12  
13 1) Charité– Universitätsmedizin Berlin, Berlin, Germany  
14  
15 2) Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Berlin, Germany  
16  
17 3) Helmholtz-Zentrum Geesthacht (HZG), Institute of Biomaterial Science, Teltow, Germany  
18  
19 4) Seoul National University, College of Veterinary Medicine and Research Institute for Veterinary Science,  
20  
21 Seoul, Republic of Korea  
22  
23 5) Berlin Institute of Health, Berlin, Germany  
24  
25 6) Max Planck Institute for Molecular Genetics, Berlin, Germany  
26  
27  
28  
29

30 **Corresponding Author:**

31  
32 Dr. Harald Stachelscheid  
33 Charite - Universitätsmedizin Berlin  
34 Berlin-Brandenburg Center for Regenerative Therapies (BCRT)  
35 Berlin Institute of Health - Stem Cell Core Facility  
36

37 Augustenburger Platz 1  
38 13353 Berlin

39  
40 Tel: +49 30 450 539428  
41 Fax: +49 30 450 524935  
42

43 Email: [Harald.Stachelscheid@charite.de](mailto:Harald.Stachelscheid@charite.de)  
44  
45  
46

47 **Key words:**

48  
49 Induced Pluripotent Stem Cells, TVA, ALV, selective cell targeting, lineage tracing  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## **Abstract**

1 Human induced pluripotent stem cells (hiPSC) differentiate into multiple cell types. Selective cell targeting is  
2  
3 often needed for analyzing gene function by overexpressing proteins in a distinct population of hiPSC-derived  
4  
5 cell types and for monitoring cell fate in response to stimuli. However, to date this has not been possible, as  
6  
7 commonly used viruses enter the hiPSC via ubiquitously expressed receptors. Here, we report for the first time  
8  
9 the application of a heterologous avian receptor, the tumor virus receptor A (TVA), to selectively transduce  
10  
11 TVA<sup>+</sup> cells in a mixed cell population. Expression of the TVA surface receptor via genetic engineering renders  
12  
13 cells susceptible for infection by avian leucosis virus (ALV). We generated hiPSC lines with this stably  
14  
15 integrated, ectopic TVA receptor gene that expressed the receptor while retaining pluripotency. The  
16  
17 undifferentiated hiPSC<sup>TVA+</sup> as well as their differentiated progeny could be infected by recombinant ALV (so-  
18  
19 called RCAS virus) with high efficiency. Due to incomplete receptor blocking, even sequential infection of  
20  
21 differentiated or undifferentiated TVA<sup>+</sup> cells was possible.

22  
23  
24 In conclusion, the TVA/RCAS system provides an efficient and gentle gene transfer system for hiPSC and  
25  
26 extends our possibilities for selective cell targeting and lineage tracing studies.  
27

## Introduction

1  
2 Induced pluripotent cells (iPSC) have the capacity for unlimited self-renewal *in vitro* and for differentiation into  
3 nearly every cell type of the mammalian body [20]. As such, differentiated cells derived from human iPSC  
4 (hiPSC) are ideally suited for mode-of-action-analyses, the establishment of disease models or pharmacological  
5 screenings[4]. Most of these applications involve genetic engineering to introduce transgenes like reporter  
6 constructs or overexpression cassettes to study e. g. the influence of specific genes on cell differentiation or for  
7 establishing reporter cell lines. Here, besides transfection, transduction is often considered an alternative  
8 technology as it avoids many of the stress triggers usually associated with chemical and physical transfection  
9 methods. It is currently not easily possible to selectively target specific subpopulations of cells for transfection or  
10 transduction. The ability to efficiently transduce only specific subpopulations of cells would be of benefit  
11 especially for stem cells and their differentiated progeny for example to trigger transgene expression and monitor  
12 cell fate of only the targeted cell types in a mixed cell population.  
13

14 For overexpression studies in avian cells the avian replication competent ALV long terminal repeat (LTR) with a  
15 splice acceptor (RCAS) virus is a well-established tool (Figure 1A) [1, 11]. The virus enters avian cells via  
16 interaction with the tumor virus surface receptor (TVA) that is not present in mammalian cells (Figure 1B). It has  
17 been shown that the range of host cell lines for RCAS virus can be expanded to non-avian cell lines by TVA  
18 receptor expression [2, 14]. Aside from its high transduction efficiency, recombinant RCAS virus technology  
19 enables the selective targeting of TVA receptor positive (TVA<sup>+</sup>) cells and their tracing in a mixed cell  
20 population. Additionally, it is possible to transfer multiple genes, located on different viral vectors through co-  
21 infection [7] as well as by sequential infection, due to impaired TVA receptor blocking by the RCAS virus in  
22 infected mammalian TVA<sup>+</sup> cells [22].  
23

24 There have been several studies reporting the application of this TVA-RCAS system in non-avian organisms,  
25 mostly in mice [5, 21]. In 1994, Federspiel *et al.* were the first to generate a transgenic mouse, in which the TVA  
26 receptor was under the control of the promoter of the chicken gene skeletal muscle  $\alpha$ -actin[5]. Thus, the TVA  
27 receptor was only expressed in skeletal muscle cells, rendering them susceptible for selective RCAS virus  
28 infection. To date, however, the extension of the RCAS overexpression system has not been applied in human  
29 iPSC, despite its potential benefit for versatile and targeted gene transfer and cell tracing of hiPSC<sup>TVA+</sup> and their  
30 progeny.  
31

32 The exceptional properties of the RCAS overexpression system exploits the distant genetic origin of the RCAS  
33 virus compared to other viruses usually used when working with hiPSC. The RCAS virus is a member of the  
34

1 avian leucosis and sarcoma virus (ALSV) family. In this study, the replication competent avian leukoma virus  
2 long terminal repeat (LTR) with a splice acceptor with bryan high-titer polymerase (BP) virus (RCASBP(A)),  
3 created by Hughes and colleagues [12], has been used. The RCASBP(A) virus is assigned to group A, because it  
4 expresses the envelope A protein (envA), enabling it to bind specifically to the TVA receptor. To facilitate  
5 overexpression studies, the oncogene virus-sarcoma (v-src) gene in this viral vector has been replaced by a  
6 multiple cloning site (MCS), allowing insertion of cDNA sequences with a length of up to 2.8 kb. The gene of  
7 interest is transcribed from the viral LTR. There is also another version of this RCASBP(A) virus that has no  
8 splice acceptor, which is called replication competent ALV LTR with no splice acceptor with BP virus  
9 (RCANBP(A)). Here, the transgene expression is not driven by the LTR and to get a reliable expression of the  
10 gene of interest, introduction of a heterologous promoter into the viral genome is required.

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21 The aim of the study was to apply the TVA-RCAS system to hiPSC, implementing the versatility of this gene  
22 transfer technology to human stem cell research. For that, we introduced the gene coding the avian TVA receptor  
23 into the genome of a hiPSC line using transposon technology (Figure 1C) [6, 13]. Stable hiPSC<sup>TVA+</sup> clones  
24 remained pluripotent and the RCAS virus was able to infect both, undifferentiated cells as well as their  
25 differentiated progeny.  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## **Methods:**

### **Cell culture**

Human iPSCs (IMR90-4, WiCell, Madison, US) and hiPSC<sup>TVA+</sup> were cultured in TeSR E8 medium (StemCell Technologies, Cologne, Germany) supplemented with 100 UI/ml of each penicillin/streptomycin (Lonza, Basel, Switzerland) in 6 well dishes (Costar, Washington, DC, US) coated with Geltrex (Gibco, Paisley, UK) and routinely passaged using Gentle Cell Dissociation Reagent (StemCell Technologies) every 5-7 days at a splitting ratio of ~1:6.

### **TVA receptor expression vector cloning**

A myc-tagged TVA950 [19] has been cloned into a modified pPB-CAG.EBNXN plasmid [24]. Briefly, the PiggyBac transposon vector used contains a human EF1 $\alpha$  promoter driving transgene expression and an additional puromycin resistance cassette. Cloning details and vector sequence are available on request.

### **Transfection and selection of hiPSC<sup>TVA+</sup>**

For transfection, hiPSC were dissociated into a single cell suspension by treatment with accutase (Gibco) followed by washing and resuspension in TeSR E8 medium containing 10 nM ROCK inhibitor Y26732 (Wako Chemicals, Neuss, Germany). Transfection was carried out using the Neon Transfection System (Invitrogen, Carlsbad, US). Briefly,  $1 \times 10^5$  cells were transfected with 0,5  $\mu$ g total DNA consisting of PB TVA950 transposon and PB transposase [24] in a ratio of 10:1 (w/w), using 10 $\mu$ l tips, settings 1.200 V for 30 ms, 1 pulse. After transfection, cells were seeded in Geltrex coated culture dishes in TeSR E8 medium. 10 nM ROCK inhibitor was added for the first 24h of culture. After six days, stably transfected cells were selected by addition of 1  $\mu$ g/ml puromycin (Invivogen, Toulouse, France) to the culture medium.

Single cell clones were isolated using the CloneStem Kit (Biolamina, Sundbyberg, Sweden), following the manufacturer's instructions.

### **Immunofluorescence**

hiPSC<sup>TVA+</sup> were cultured on 96 well imaging plates (CellCarrier, Perkin Elmer, Waltham, US) in TeSR E8 for three days. For detection of the extracellular Myc-tag attached to the TVA receptor, cells were fixed with Cytofix (BD, San Jose, US), blocked with blocking buffer (10 % FCS superior (Biochrom, Berlin, Germany) in PBS (Lonza)) and then incubated with anti-Myc-tag antibody (Cell Signaling, Leiden, Netherlands; dilution 1:2000). The cells were washed with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG secondary

antibody (Invitrogen, 1:2000). Nuclei were stained with Hoechst 33342 (2,5 µg/ml in PBS; Life Technologies, Grand Island, NY, US).

Staining of pluripotency markers was performed by fixation with Cytotfix reagent, followed by blocking and permeabilization with 1X PermWash reagent (both BD) supplemented with 5% chicken serum (Life Technologies). Thereafter, cells were incubated using anti-Nanog rabbit mAb (Cell Signaling, 1:400), followed by secondary antibody anti-rabbit Alexa Fluor594 (Life Technologies). Then, staining with the fluorescence dye-conjugated antibodies octamer transcription factor 3/4 (Oct3/4) (PerCP-Cy7, BD, 1:100), stage specific embryonic antigen 4 (SSEA4) (FITC, R&D, Minneapolis, US; 1:100) and tumor rejection antigen 1-81 (Tra-1-81) (DyLight 650, Novus Biologicals, Littleton, US; 1:100) was performed. Nuclei were stained with Hoechst 33342 (2,5 µg/ml in PBS).

Microscopic analysis was performed with either a 2 Photon microscope (LEICA SP5 confocal microscope equipped with a Mai Tai HP multiphoton laser), or an Operetta imaging system (Perkin Elmer), as indicated.

#### **RCASBP(A) and RCANBP(A) cloning and production**

RCASBP(A)/RCANBP(A) vectors were obtained from Stephen Hughes. Insertion of GFP, RFP, monomeric Cherry (mCherry) and the simian virus 40(SV40) promoter (Addgene accession numbers #17619, #20972, #18807, Cambridge, US) was done by performing Gibson Assembly (NEB, Hitchin, UK; described in [8]). Primers were purchased from Eurofins (Berlin, Germany, Supplemental Table 1). RCASBP(A)/RCANBP(A) viruses were produced in DF1 cells (ATCC, Manassas, US) after transfection of the viral plasmids with PEI (Polysciences Inc., Eppelheim, Germany). On three consecutive days viral particles were harvested by collecting the supernatant, followed by ultracentrifugation for high-titer concentrates. Titers were determined as plaque forming units (PFU/ml) one day after DF1 cell infection[15].

#### **Infection of hiPSC TVAs**

For RCAS infection, hiPSC<sup>TVA+</sup> were cultured in 24 well plates (TPP, Trasadingen, Switzerland) or 96 well plates. Viral transduction was performed at a MOI of 50. Medium was changed every day. 2-3 days post infection hiPSC<sup>TVA+</sup> were analyzed after live cell staining for nuclei (Hoechst 33342 in medium, 2,5 µg/ml in PBS) using an Operetta imaging system. Sequential infection was done 3 days apart.

#### **Fluorescence activated cell sorting (FACS) analysis**

Cells were dissociated using Gentle Cell Dissociation Reagent and fixed with Cytotfix. For Myc-tag quantification, cells were stained with anti-Myc-tag antibody (Cell Signaling, 1:2000), washed with PBS,

1 followed by staining with secondary antibody Alexa Fluor 488 goat anti-mouse IgG secondary antibody  
2 (Invitrogen, 1:2000). Afterwards, cells were diluted in FACS buffer, consisting of 2 % bovine serum albumin  
3 (Sigma Aldrich, Seelze, Germany) and 2 mM EDTA (Sigma Aldrich) in PBS. To quantify transduction, cells,  
4 diluted in FACS buffer, were directly used for FACS analysis, which was performed with an Accuri C6 (BD,  
5 San Jose, US). Data was analyzed with the FCS express 4 software (De Novo, Los Angeles, US).  
6  
7  
8  
9

### 10 **Undirected differentiation of hiPSC in 2D and 3D culture**

11 Undirected differentiation of hiPSC<sup>TVA+</sup> in 2D culture was induced by culture in DMEM Low Glucose  
12 (Biochrom), 10 % FCS superior, 2mM/ml L-Glutamine (Biochrom) and 100 UI/ml Penicillin/Streptomycin  
13 (Biochrom) for three days, following immune staining and FACS analysis.  
14  
15  
16  
17

18 To differentiate hiPSC<sup>TVA+</sup> in 3D culture, embryoid bodies (EBs) were generated either by cell aggregation in  
19 ultra low attachment plates (Costar) or in AggreWell plates (StemCell Technologies). Subsequently, after  
20 aggregate formation, EBs were cultured either in DMEM Low Glucose supplemented with 10 % FCS superior,  
21 2mM/ml L-Glutamine and 100 UI/ml Penicillin/Streptomycin for 10 days or in KnockOut DMEM/F12  
22 (Invitrogen) supplemented with 20 % KnockOut Serum Replacer (Invitrogen), 50 µM 2-Mercaptoethanol  
23 (Invitrogen), 1X MEM Non-Essential Amino Acids (Invitrogen), 2mM/ml L-Glutamine and 100 UI/ml  
24 Penicillin/Streptomycin for 7 days. Medium was changed every other day.  
25  
26  
27  
28  
29  
30  
31  
32  
33

### 34 **Splinkerette PCR**

35 A Splinkerette PCR was performed according to Potter and Luo 2014[18] to determine copy number and  
36 chromosomal integration site of the PB transposon. In short: genomic DNA was isolated using the Genomic  
37 DNA from tissue kit (Machery-Nagel, Dueren, Germany). 1 µg of this gDNA was digested with BstYI  
38 (Fermentas, Burlington, Canada) for 2 h, followed by ligation of the resulting DNA fragments to Splinkerette  
39 oligos (sequences according to [18], Supplemental Table 1). Two rounds of PCR were conducted, amplification  
40 products separated using an 1 % agarose gel (Invitrogen) and bands indicating single-copy events were purified  
41 with the Omnipure OLS kit (Omni Life Science, East Taunton, US). These PCR products then were sequenced  
42 using a 3730 DNA Analyzer (Life Technologies; primer sequences are listed in Supplemental Table 1).  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



### RNA isolation and quantitative PCR analysis of marker genes

1  
2 Undifferentiated iPSC<sup>TVA+</sup> and EBs were harvested and disrupted using a Shredder (Qiagen, Valencia, US),  
3  
4 followed by RNA extraction (RNeasy Plus Mini Kit, Qiagen). 1 µg of total RNA were reverse transcribed into  
5  
6 cDNA with the TaqMan Reverse Transcription Reagents (Invitrogen). After confirming RNA integrity by 2 %  
7  
8 agarose gel electrophoresis, cDNA was applied for quantitative analysis of trilineage differentiation potential  
9  
10 performing quantitative PCR analysis using commercially available Taqman probes and measured on a  
11  
12 QuantStudio 6 machine (Life Technologies, Supplemental Table 2). For normalization the expression of the  
13  
14 housekeeping genes  $\beta$ -Actin, Transcriptional repressor CTCF, E1A binding protein p300 and Mothers against  
15  
16 decapentaplegic homolog 1 (Smad1) were determined. To obtain the  $\Delta$ Ct values of the genes of interest (GOI),  
17  
18 the mean of the housekeeping genes (ranging between 23 and 25) was subtracted from their Ct value. The  $\Delta\Delta$ Ct  
19  
20 value was calculated by  $\Delta$ Ct (GOI) minus  $\Delta$ Ct of the reference values of undifferentiated hiPSC<sup>TVA+</sup>.  
21  
22

### Statistics

23  
24  
25 Statistical analysis for standard derivation of RCAS infection of hiPSC<sup>TVA+</sup> and its differentiated progeny was  
26  
27 calculated using Prism 6 software (GraphPad, La Jolla, USA). Groups consisted of three independent  
28  
29 experiments each.  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## **Results:**

### **Ectopic TVA receptor is located on the cell surface in human iPSC**

hiPSC were transfected with a PiggyBac transposon carrying the TVA receptor under the control of the housekeeping promoter elongation factor 1 (EF1). Transgene-positive cells were isolated by antibiotic selection, cloned and analyzed for single transposon integration.

TVA receptor expression in the three isolated hiPSC<sup>TVA+</sup> clones was analyzed by immunofluorescence staining of the Myc-tag fused to the extracellular c-terminus of the TVA receptor protein.

Using 2 photon microscopy, expression of the TVA receptor on the cell surface of hiPSC<sup>TVA+</sup> was shown (Figure 2A+C). FACS analysis was performed and revealed the presence of the TVA receptor in 93.31 % of the cells (Figure 2B).

### **hiPSC<sup>TVA+</sup> are susceptible to RCAS virus infection**

To analyze the efficiency by which hiPSC<sup>TVA+</sup> and their differentiated progeny can be infected by RCAS virus, hiPSC<sup>TVA+</sup> were infected with RCASBP(A) carrying the green fluorescent protein (GFP) gene as reporter. No viral reporter gene expression could be observed in undifferentiated hiPSC<sup>TVA+</sup> three days post infection (Figure 3D). Interestingly, after spontaneous differentiation of RCASBP(A) GFP-transduced hiPSC<sup>TVA+</sup>, viral gene expression was initiated as evidenced by GFP expression (Figure 3A). When differentiated cells derived from the hiPSC<sup>TVA+</sup> were infected with RCASBP(A), GFP expression was observed 72 hours after infection (Figure 3B). In these experiments, high infection efficiencies were observed by FACS analysis, specifically 73.3 % ( $\pm$  7.6 %) for undifferentiated cells and 64.0 % ( $\pm$  7.2 %) for differentiated cells (Figures 3E, F).

To test the influence of the specific promoter on the transgene expression in undifferentiated hiPSC, an alternative promoter to the viral LTR for initiation of the GFP reporter gene expression was tested. When hiPSC<sup>TVA+</sup> were infected with a RCANBP(A) carrying GFP under SV40 promoter control GFP expression was detected 72 hours post infection (Figure 3C). FACS analysis showed an infection efficiency of 56.0 % ( $\pm$  13.1 %) (Figure 3G). GFP expression was retained upon cell differentiation (data not shown).

### **hiPSC<sup>TVA+</sup> are pluripotent and have a single transposon integration site**

For most conceivable applications of the hiPSC<sup>TVA+</sup> technology, it is mandatory to confirm the continued pluripotency of the cells after the genetic engineering by introduction of the TVA cDNA. Pluripotency of the hiPSC<sup>TVA+</sup> was confirmed by detection of the expression of pluripotency markers in undifferentiated cells and

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

evaluation of the differentiation capability into cells of all three germ layers. First, co-expression of the pluripotency markers SSEA4, TRA-1-81, Oct3/4 and NANOG in undifferentiated hiPSC<sup>TVA+</sup> was confirmed by positive immunofluorescence staining (Figure 4). Second, gene expression was analyzed in undifferentiated hiPSC<sup>TVA+</sup> and embryoid bodies (EBs) by quantitative RT-PCR using markers of all three germ layers. In the EBs the expression of the ectodermal markers Paired box protein 3 (PAX3), Myosin III B (MYO3B) and Wingless-Type MMTV Integration Site Family, Member 1 (WNT1), the endodermal markers Krueppel-like factor 5 (KLF5), SRY (sex determining region Y)-box 17(SOX17) and Hepatocyte nuclear factor 4-alpha (HNF4A) as well as the mesodermal markers Heart- and neural crest derivatives-expressed protein 1(HAND1), HAND2 and Homeodomain-only protein (HOPX) was detected. In parallel, the pluripotency markers POU domain, class 5, transcription factor 1(POU5F1/Oct 4), Nanog and sex determining region Y box 2(Sox 2) were down regulated (Supplemental Figure 1).

Splinkerette PCR was performed to examine the number and position of integration sites of the PB transposon . For the hiPSC<sup>TVA+</sup> clone#1 used mostly throughout the investigation reported here, intra-intronic integration into the *zinc finger matrin-type protein 4 (ZMAT4)* gene was determined (Table 1).

#### **Infection of human cells with RCAS vectors is highly specific to TVA expressing cells**

Next we tested the application for the selective targeting of hiPSC<sup>TVA+</sup> in mixed cell populations.

As a proof of concept hiPSC<sup>TVA+</sup> were co-cultured with a stable hiPSC<sup>GFP+</sup> cell line. This mixed population was infected with a RCANBP(A) virus, containing mCherry under the control of the SV40 promoter. Microscopic analysis three days after infection revealed that expression of the fluorescent proteins on the cellular level was mutually exclusive. In consequence, only GFP-negative hiPSC<sup>TVA+</sup> were infected by the mCherry encoding RCAN virus, as evident from cell clusters of the respective hiPSC subpopulations growing into each other (Figure 5).

Moreover, sequential infection with two RCAS viruses at different time points was possible. This has been tested in hiPSC<sup>TVA+</sup> (Data not shown), as well as their differentiated progeny (Figure 5B). Here, hiPSC<sup>TVA+</sup> were first infected with a RCAS RFP virus, followed by infection with a RCAS GFP virus three days later.

## **Discussion:**

1  
2 The aim of this study was to establish the avian leukosis virus (ALV)-based retroviral vector TVA-RCAS system  
3 for human iPSC, expanding its reach to stem cell research. To establish and evaluate the TVA-RCAS system in  
4 stem cells, we generated hiPSC clones expressing the TVA receptor gene, evaluated the presence and  
5 functionality of the receptor, confirmed their continued pluripotency and lastly characterized the potency of the  
6 system by determining the infection efficiency and selectivity in a mixed cell population using RCAS viral  
7 vectors.  
8  
9

10  
11  
12  
13  
14 In our study we were able to stably transfect and express the TVA receptor in hiPSC using a PiggyBac  
15 transposon, taking advantage of the high chromosomal integration activity of this gene transfer system[23].  
16  
17 There was no obvious effect on pluripotency subsequent to the genetic manipulation of the hiPSC. As previously  
18 described in the literature, the EF1 promoter proved to be suitable for high ectopic gene expression in hiPSC, as  
19 well as in their differentiated progeny [9, 16]. Correct localization of the TVA receptor on the cell surface and  
20 susceptibility of hiPSC<sup>TVA+</sup> for the RCAS virus were verified. However, viral gene expression in hiPSC<sup>TVA+</sup>  
21 turned out to be problematic as RCAS-vector based GFP-transgene expression was not detected in hiPSC<sup>TVA+</sup>  
22 after infection, when using the viral LTR for gene overexpression (Figure 3D). One explanation could be that the  
23 hiPSC are not permissive for the RCAS virus. This possibility was ruled out by showing that LTR-driven GFP  
24 transgene expression started after differentiation of hiPSC<sup>TVA+</sup> infected with a RCAS vector. Another  
25 explanation could be the lack of functionality of the viral LTR in hiPSC. Our study suggests that this is the case  
26 as we were able to overcome this problem by using the heterologous SV40 promoter in a modified RCAS  
27 version, the RCAN virus.  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41

42 We also tested the EOS-C 3+ (Early transposon promoter and Oct4 and Sox2 enhancers) [10], Thymidine Kinase  
43 (TK) [17] and CMV promoters, which were functional in initiating ectopic gene expression in hiPSC, but gene  
44 expression was lower compared to SV40 (data not shown).  
45  
46  
47  
48

49 When using the EF1 and Cytomegalovirus (CMV) early enhancer/chicken beta actin (CAG) promoters, there  
50 was no functional RCAN virus production even in the DF1 production cell line. We hypothesize that this is due  
51 to intron sequences being recognized and spliced before integration into the hiPSC genome, which leads to a  
52 non-functional shortened promoter version.  
53  
54  
55  
56  
57

58 Nevertheless, we observed higher infection efficiencies in differentiated hiPSC<sup>TVA+</sup> progeny than in hiPSC<sup>TVA+</sup>.  
59 This might be explained by increased silencing of retroviral DNA in hiPSC after integration into the genome.  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Whatever the mechanism, the RCAS vector system introduced here, provides not only the opportunity for infection with at least two different viral constructs, but also for multiple re-infection at later time points. This enhances the utility of the RCAS viruses for e.g. lineage tracing. The unusual possibility for subsequent transductions is most likely due to incomplete blocking of the TVA receptors by EnvA protein on the cell surface, since there is no sufficient viral protein production in non-avian cells[22].

The final purpose of this study was to confirm selectivity of RCASBP(A) infection towards hiPSC<sup>TVA+</sup> in a mixed cell population, which could be shown accurately, when co-culturing hiPSC<sup>TVA+</sup> with hiPSC stably expressing GFP followed by infection with a RCANBP(A) SV40 mCherry virus. This highlights one uniqueness of this newly designed tool: most of the commonly used retro- and lentiviral vector systems used for stem cell transduction enter cells by interactions of the vesicular stomatitis virus glycoprotein (VSV-G protein) and the corresponding receptor, which is ubiquitously present on mammalian cell membranes and therefore makes selective infection impossible[3]. In contrast to that, RCAS virus infection is guarded by the EnvA protein [11] recognizing the TVA receptor. Therefore, cellular selectivity for the RCAS virus is given, after ectopic TVA receptor expression [2, 5].

Taken together, a novel tool for the efficient transduction of hiPSC was developed. We foresee the selective targeting of defined subpopulations of cells, either in mixed stem cell cultures or in lineage tracing experiments, as well as possible sequential infection as main areas of application for this technology.

### **Conclusion:**

In this study we report for the first time the successful application of the TVA-RCAS system for efficient infection of hiPSC. It represents a promising new avenue for generating genetically engineered stem cells via viral transduction for differentiation, cell fate and gene function studies. The TVA-RCAS system has the added value of selective targeting hiPSC<sup>TVA+</sup> in mixed populations as well as sequential infection with different viruses.

### **Acknowledgement**

We thank Mareen Schmidt-von Kegler and Maria Walther as well as the Berlin Institute of Health (BIH) Stem Cell Core Facility for excellent technical assistance and the people from the laboratory of Prof. Petra Seemann and Dr. Andreas Kurtz for helpful discussions regarding the study. Additionally, we thank Dr. Stephen Hughes for kindly providing us with the RCAS and RCAN vectors and Dr. Ansgar Petersen for help at the 2 photon microscope. We appreciate the financial support and provision of laboratory facilities by Prof. Petra Reinke.

Laura Hildebrand is a member of the DFG funded Berlin-Brandenburg School for Regenerative Therapies GSC

203. This work was supported by the grant 1315848A for the Berlin-Brandenburg Center for Regenerative Therapies (BCRT) by the Initiative and Networking Fund of the Helmholtz Association, Germany.

**Conflict of interest**

All authors state that they have no conflict of interest.

## References:

1. Barnard RJ, Elleder D, Young JA (2006) Avian sarcoma and leukosis virus-receptor interactions: from classical genetics to novel insights into virus-cell membrane fusion. *Virology* 344:25-29
2. Brennand KJ, Simone A, Jou J et al. (2011) Modelling schizophrenia using human induced pluripotent stem cells. *Nature* 473:221-225
3. Cronin J, Zhang XY, Reiser J (2005) Altering the tropism of lentiviral vectors through pseudotyping. *Current gene therapy* 5:387-398
4. Csobonyei M, Polak S, Koller J et al. (2014) Induced pluripotent stem cells and their implication for regenerative medicine. *Cell Tissue Bank*
5. Federspiel MJ, Bates P, Young JA et al. (1994) A system for tissue-specific gene targeting: transgenic mice susceptible to subgroup A avian leukosis virus-based retroviral vectors. *Proceedings of the National Academy of Sciences of the United States of America* 91:11241-11245
6. Fraser MJ, Smith GE, Summers MD (1983) Acquisition of Host Cell DNA Sequences by Baculoviruses: Relationship Between Host DNA Insertions and FP Mutants of *Autographa californica* and *Galleria mellonella* Nuclear Polyhedrosis Viruses. *J Virol* 47:287-300
7. Fusaki N, Ban H, Nishiyama A et al. (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* 85:348-362
8. Gibson DG, Young L, Chuang RY et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6:343-345
9. Hong S, Hwang DY, Yoon S et al. (2007) Functional analysis of various promoters in lentiviral vectors at different stages of in vitro differentiation of mouse embryonic stem cells. *Mol Ther* 15:1630-1639
10. Hotta A, Cheung AY, Farra N et al. (2009) Isolation of human iPS cells using EOS lentiviral vectors to select for pluripotency. *Nat Methods* 6:370-376
11. Hughes SH (2004) The RCAS vector system. *Folia Biol (Praha)* 50:107-119
12. Hughes SH, Greenhouse JJ, Petropoulos CJ et al. (1987) Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J Virol* 61:3004-3012
13. Kim A, Pyykko I (2011) Size matters: versatile use of PiggyBac transposons as a genetic manipulation tool. *Mol Cell Biochem* 354:301-309
14. Lewis BC, Chinnasamy N, Morgan RA et al. (2001) Development of an avian leukosis-sarcoma virus subgroup A pseudotyped lentiviral vector. *J Virol* 75:9339-9344
15. Morgan BA, Fekete DM (1996) Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol* 51:185-218
16. Norrman K, Fischer Y, Bonnamy B et al. (2010) Quantitative comparison of constitutive promoters in human ES cells. *PLoS One* 5:e12413
17. Ou W, Li P, Reiser J (2013) Targeting of herpes simplex virus 1 thymidine kinase gene sequences into the OCT4 locus of human induced pluripotent stem cells. *PLoS One* 8:e81131
18. Potter CJ, Luo L (2010) Splinkerette PCR for mapping transposable elements in *Drosophila*. *PLoS One* 5:e10168
19. Rong L, Gendron K, Bates P (1998) Conversion of a human low-density lipoprotein receptor ligandbinding repeat to a virus receptor: identification of residues important for ligand specificity. *Proceedings of the National Academy of Sciences of the United States of America* 95:8467-8472

- 1
  - 2
  - 3
  - 4
  - 5
  - 6
  - 7
  - 8
  - 9
  - 10
  - 11
  - 12
  - 13
  - 14
  - 15
  - 16
  - 17
  - 18
  - 19
  - 20
  - 21
  - 22
  - 23
  - 24
  - 25
  - 26
  - 27
  - 28
  - 29
  - 30
  - 31
  - 32
  - 33
  - 34
  - 35
  - 36
  - 37
  - 38
  - 39
  - 40
  - 41
  - 42
  - 43
  - 44
  - 45
  - 46
  - 47
  - 48
  - 49
  - 50
  - 51
  - 52
  - 53
  - 54
  - 55
  - 56
  - 57
  - 58
  - 59
  - 60
  - 61
  - 62
  - 63
  - 64
  - 65
20. Takahashi K, Tanabe K, Ohnuki M et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861-872
21. Vervoort VS, Lu M, Valencia F et al. (2008) A novel Flk1-TVA transgenic mouse model for gene delivery to angiogenic vasculature. *Transgenic research* 17:403-415
22. Von Werder A, Seidler B, Schmid RM et al. (2012) Production of avian retroviruses and tissue-specific somatic retroviral gene transfer in vivo using the RCAS/TVA system. *Nature protocols* 7:1167-1183
23. Wilson MH, Coates CJ, George AL, Jr. (2007) PiggyBac transposon-mediated gene transfer in human cells. *Mol Ther* 15:139-145
24. Yusa K, Rad R, Takeda J et al. (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods* 6:363-369



**Figure legends:**

**Figure 1: Scheme of the TVA-RCAS system in avian and mammalian cells** The genome of the RCAS virus consists of two Long Terminal Repeats (LTR) flanking the three viral genes (1) group specific antigen (gag) (2) polymerase (pol) (3) envelope (env) and the multiple cloning site (MCS). [A] In avian cells the RCASBP(A) virus enters through the TVA receptor and is replication competent. [B] In mammalian cells the virus cannot enter, as the TVA receptor is not expressed. [C] By transfecting a transposon holding the TVA receptor cDNA, infection of mammalian cells becomes possible; however, no functional RCASBP(A) virus is produced.

**Figure 2: Immunolocalisation of TVA receptor in hiPSC<sup>TVA+</sup>** [A] Anti-Myc-tag stained hiPSC<sup>TVA+</sup> for detection of TVA receptor expression (left panel). Merge of Hoechst33342 and Myc-tag staining and view of the xz and yz plane (right panel). [B] Quantification of TVA receptor using FACS analysis. [C] Magnification of Hoechst33342 and Myc-Tag staining. (Scale bar = 61  $\mu$ m)

**Figure 3: Detection of RCASBP(A) and RCANBP(A) infection in hiPSC<sup>TVA+</sup>** [A] GFP expression in differentiated hiPSC<sup>TVA+</sup> progeny infected in an undifferentiated state (left panel). Merge of Hoechst33342 and GFP(right panel). [B] GFP expression in differentiated hiPSC<sup>TVA+</sup> progeny infected in a differentiated state(left panel). Merge of Hoechst33342 and GFP (right panel). [C] Brightfield (left panel). GFP expression in hiPSC<sup>TVA+</sup>(middle panel). Merge of Hoechst33342 and GFP (right panel). [D-G] Quantification of GFP expression by FACS. (Scale bar = 50  $\mu$ m)

**Figure 4: Expression of pluripotency markers in hiPSC<sup>TVA+</sup>** [A] Hoechst33342 stained nuclei. [B] Anti-stage-specific embryonic antigen 4 (SSEA4) staining (cell surface) [C] Anti-Tra-1-81 staining (cell surface). [D] Anti-Oct3/4 staining (nuclear) [E] Anti-Nanog staining (nuclear). (Scale bar = 50  $\mu$ m)

**Figure 5: Selective targeting in a mixed population and sequential infection of hiPSC<sup>TVA+</sup>** [A] Selective infection: Hoechst33342 stained nuclei (left panel). mCherry expression in hiPSC<sup>TVA+</sup> infected with RCANBP(A) SV40 mCherry GFP expression in stable hiPSC GFP cell line. Merge of Hoechst 33342, mCherry and GFP. [B] Sequential infection: RFP expression in spontaneously differentiated hiPSC<sup>TVA+</sup> with RCASBP(A)RFP (left panel); and RCASBP(A) GFP (middle/left panel). Merge of RFP and GFP (middle/right panel) and merge of Hoechst33342, RFP and GFP (right panel). (Scale bar = 50  $\mu$ m)

**Supplemental Figure 1: Confirmation of trilineage differentiation potential of Clone 1 hiPSC<sup>TVA+</sup>** Undirected differentiation of the hiPSC<sup>TVA+</sup> as embryoid bodies (EBs) in two different media showed upregulation of markers for the three germ layers, while markers for pluripotency were downregulated.

Figure 1  
[Click here to download Figure: Fig.1.jpg](#)

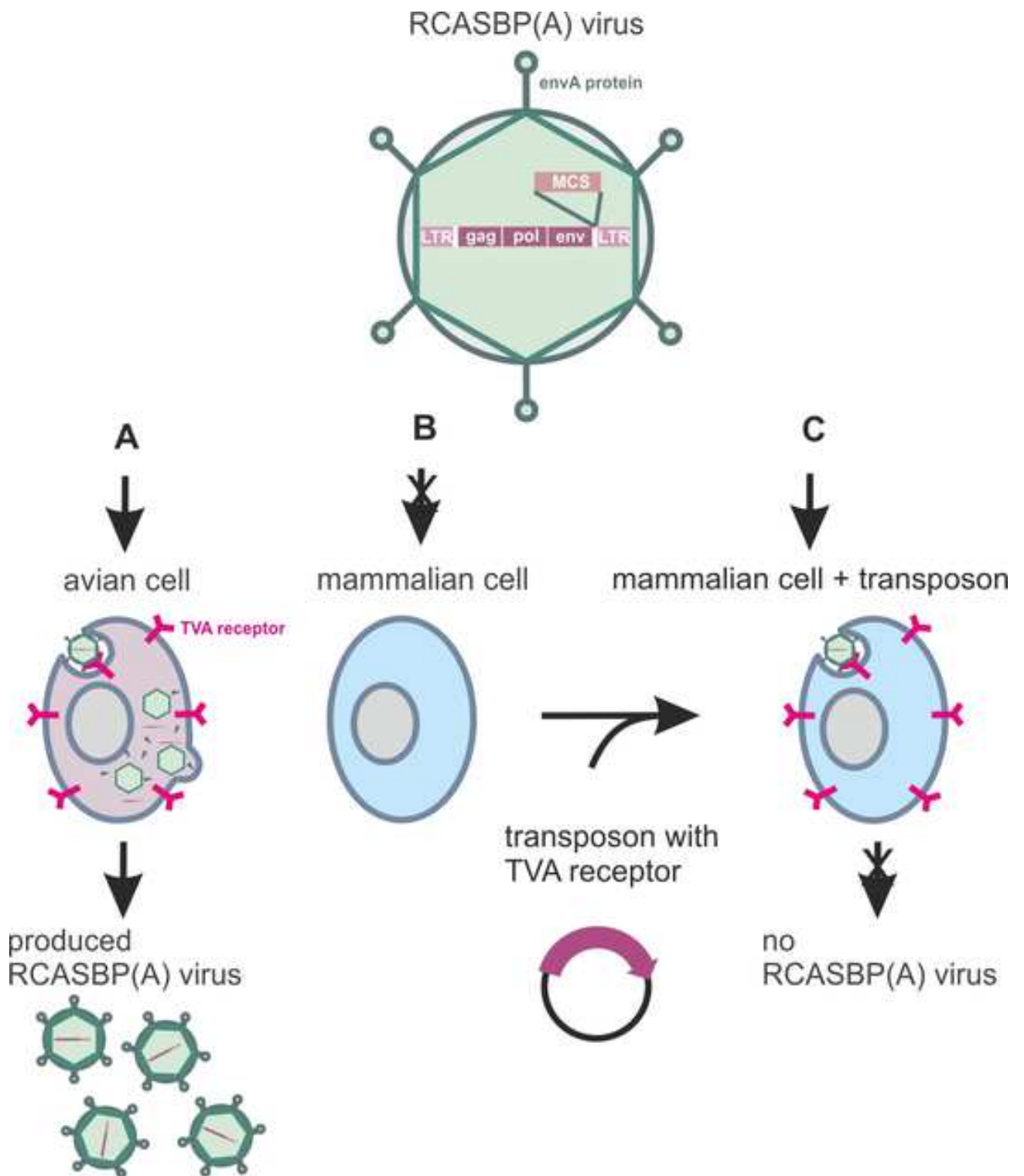


Figure 2  
[Click here to download Figure: Fig.2.jpg](#)

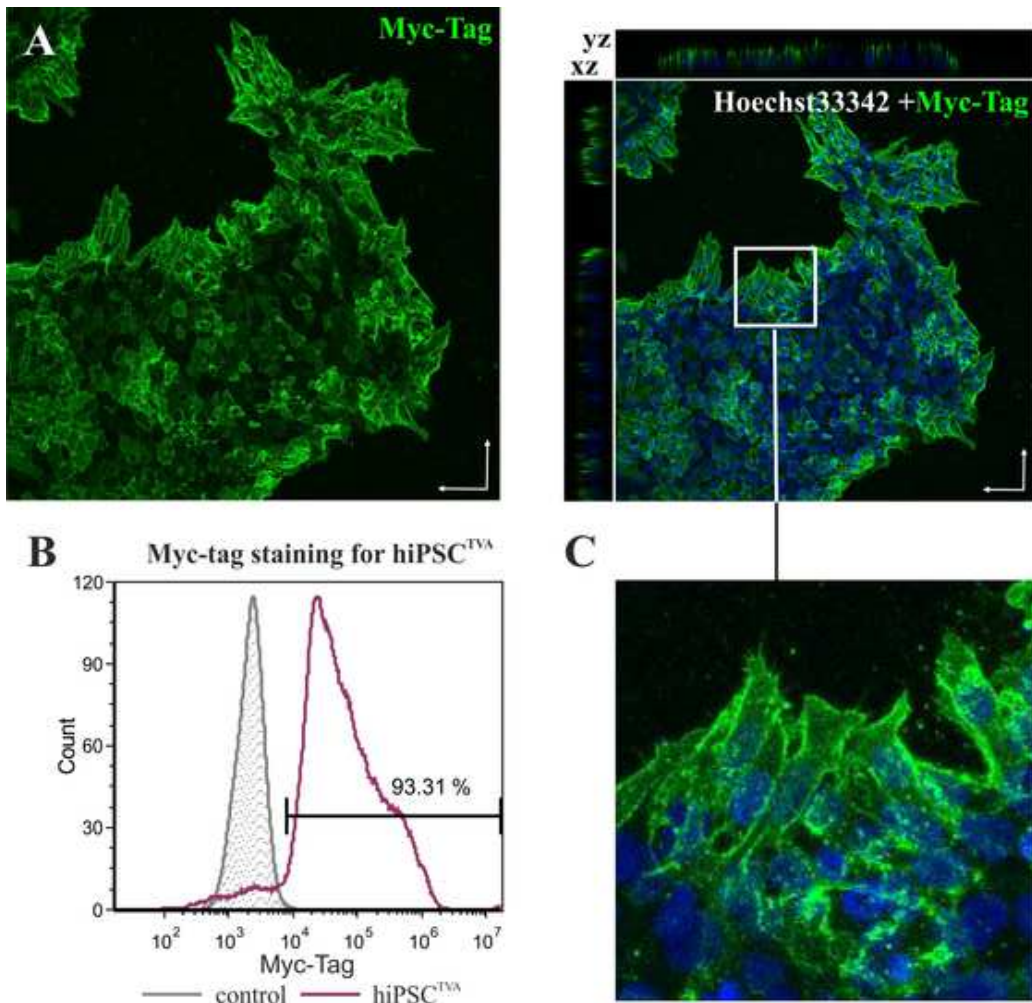


Figure 3  
[Click here to download Figure: Fig.3.jpg](#)

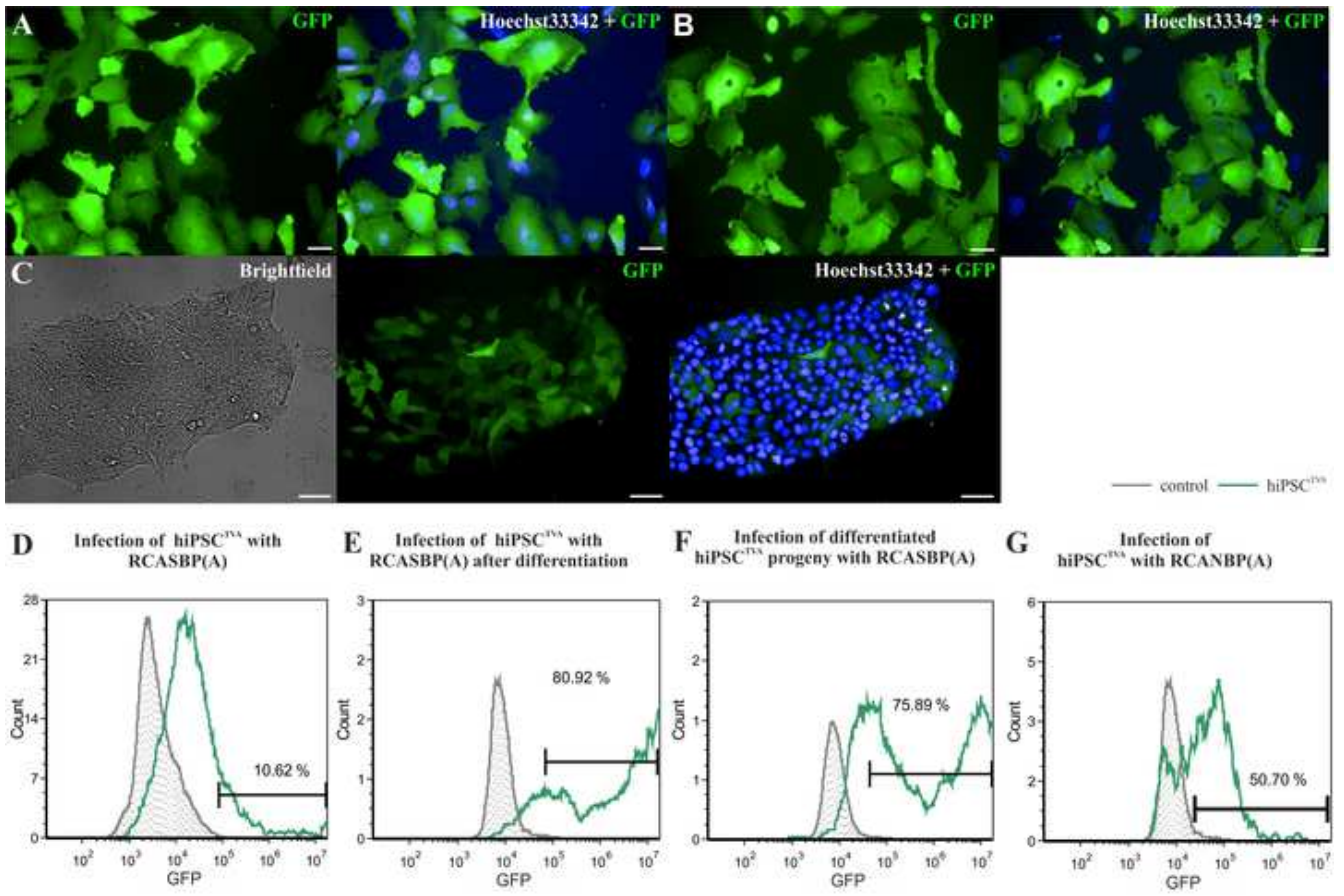


Figure 4  
[Click here to download Figure: Fig.4.jpg](#)

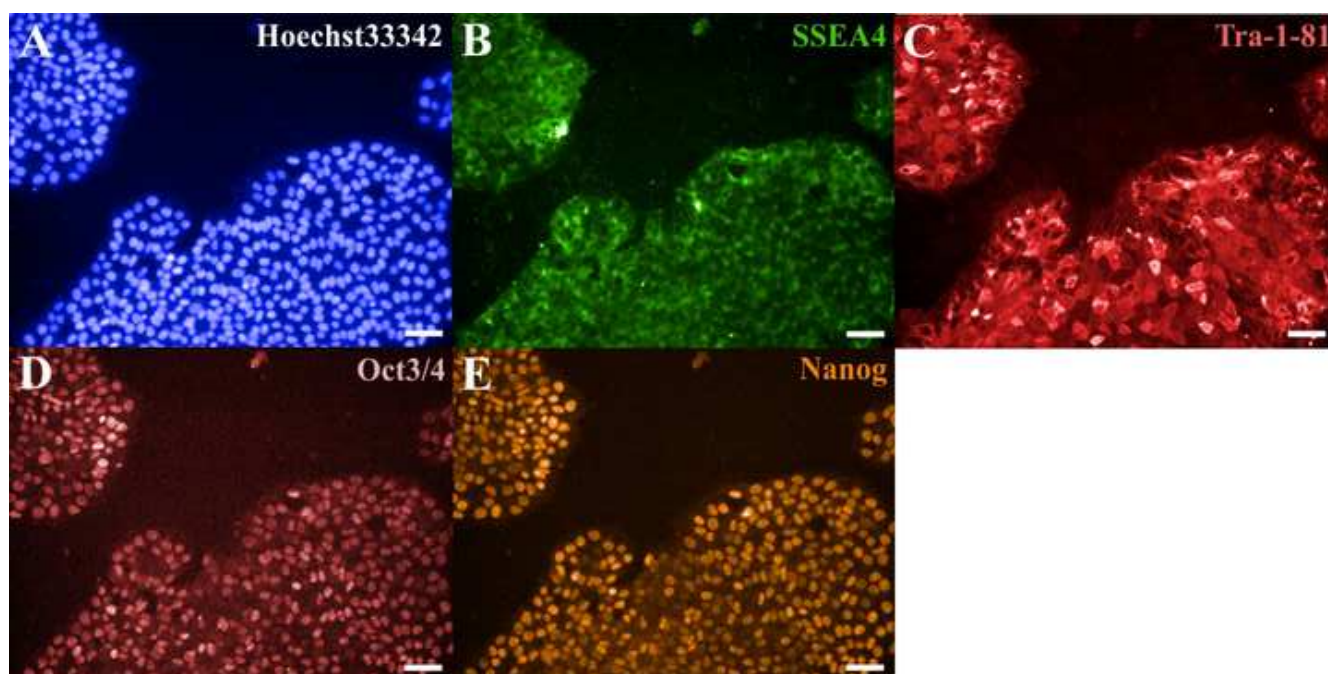




Figure 5  
[Click here to download Figure: Fig.5.jpg](#)

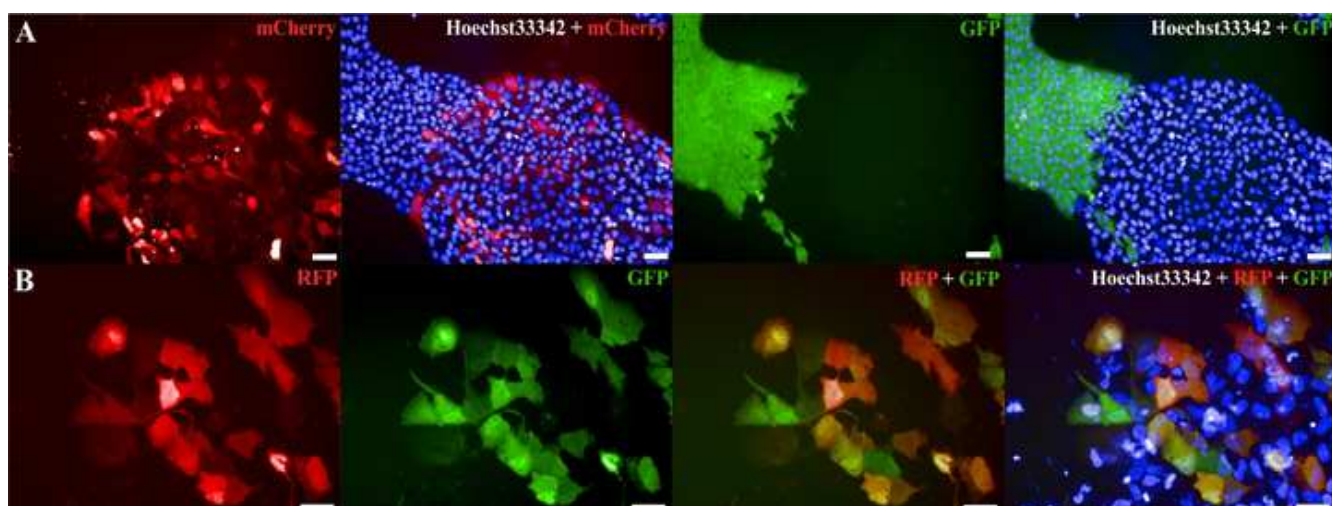


Table 1

**Table 1: Summary of the key characteristics of the generated hiPSC<sup>TVA+</sup> lineages**

Criteria		Clone 1 hiPSC <sup>TVA+a</sup>	Clone 2 hiPSC <sup>TVA+</sup>	Clone 3 hiPSC <sup>TVA+</sup>
<b>Confirmation of pluripotency</b>				
<i>Pluripotency marker expression</i>	Oct3/4 <sup>b</sup>	+ <sup>h</sup>	+	+
	Nanog	+	+	+
	SSEA4 <sup>c</sup>	+	+	+
	Tra-1-81 <sup>d</sup>	+	+	+
<i>Spontaneous Differentiation</i>	Ectoderm	+		
	Endoderm	+		
	Mesoderm	+		
<b>Transgene verification</b>				
<i>PiggyBac transposon integration sites</i>	Amount	1	1	1
	Chromosome	8	8	17
	Gene name	<i>ZMAT</i> <sup>e</sup>	<i>FOXH1</i> <sup>f</sup>	<i>KCNH6</i> <sup>g</sup>
	Gene region	Intron	Exon	Intron
<i>Staining for TVA receptor (Myc-tag)</i>	Undifferentiated	+	+	+
	Differentiated	+	+	+
<i>Transducability</i>	RCASBP(A) <sup>i</sup>	+	+	+
	RCANBP(A) <sup>j</sup>	+	+	+
<i>Viral transgene expression</i>	RCASBP(A)	-	-	-
	RCANBP(A)	<b>+</b>	<b>+</b>	<b>+</b>

<sup>a</sup> human induced pluripotent stem cells tumor receptor virus A positive (hiPSC<sup>TVA+</sup>)

<sup>b</sup> octamer transcription factor 3/4 (Oct3/4)

<sup>c</sup> stage specific embryonic antigen 4 (SSEA4)

<sup>d</sup> tumor rejection antigen 1-81 (Tra-1-81)

<sup>e</sup> Zinc finger matrin-type protein 4 (*ZMAT4*)

<sup>f</sup> Forkhead box H1 (*FOXH1*)

<sup>g</sup> Potassium voltage-gated channel subfamily H member 6 (*KCNH6*)

<sup>h</sup> + present

<sup>i</sup> RCASBP(A):replication competent Avian Leukoma Virus (ALV) Long Terminal Repeat (LTR) with a splice acceptor with Bryan high-titer polymerase

<sup>j</sup> RCANBP(A) replication competent Avian Leukoma Virus (ALV) Long Terminal Repeat (LTR) with no splice acceptor with Bryan high-titer polymerase

[Click here to download Supplementary Material: Suppl.Tab.1\\_TVA\\_CMLS.docx](#)



[Click here to download Supplementary Material: Suppl.Tab.2\\_TVA\\_CMLS.docx](#)

[Click here to download Supplementary Material: Suppl.Fig.1.jpg](#)