Video Article

# Protocol for the Direct Conversion of Murine Embryonic Fibroblasts into Trophoblast Stem Cells

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## Abstract

Trophoblast stem cells (TSCs) arise as a consequence of the first cell fate decision in mammalian development. They can be cultured *in vitro*, retaining the ability to self-renew and to differentiate into all subtypes of the trophoblast lineage, equivalent to the *in vivo* stem cell population giving rise to the fetal portion of the placenta. Therefore, TSCs offer a unique model to study placental development and embryonic versus extra-embryonic cell fate decision *in vitro*. From the blastocyst stage onwards, a distinct epigenetic barrier consisting of DNA methylation and histone modifications tightly separates both lineages. Here, we describe a protocol to fully overcome this lineage barrier by transient over-expression of trophoblast key regulators Tfap2c, Gata3, Eomes and Ets2 in murine embryonic fibroblasts. The induced trophoblast stem cells are able to self-renew and are almost identical to blastocyst derived trophoblast stem cells in terms of morphology, marker gene expression and methylation pattern. Functional *in vitro* and *in vivo* assays confirm that these cells are able to differentiate along the trophoblast lineage generating polyploid trophoblast giant cells and chimerizing the placenta when injected into blastocysts. The induction of trophoblast stem cells from somatic tissue opens new avenues to study genetic and epigenetic characteristics of this extra-embryonic lineage and offers the possibility to generate trophoblast stem cell lines without destroying the respective embryo.

## Video Link

The video component of this article can be found at https://www.jove.com/video/54277/

## Introduction

Recently, a study comparing several approaches of mouse embryonic stem cell to trophoblast stem cells conversion has revealed that in all analyzed systems, lineage conversion remained incomplete. Instead of induced trophoblast stem cells (iTSCs) so called trophoblast stem cell-like cells have been generated retaining a memory of the cell fate of origin[1]. Here, we followed a different approach of iTSC generation. Similar to the direct induction of pluripotent stem cells from murine embryonic fibroblasts (MEFs) [2], iTSCs have been directly converted from differentiated somatic tissue. First, we identified 12 candidate factors inducing TSC fate when overexpressed in MEFs. Later on, the factors Tfap2c, Gata3, Eomes and Ets2 have been identified to be necessary and sufficient for the iTSC induction [3]. Simultaneously, another group independently found Tfap2c, Gata3 and Eomes to be sufficient to convert MEFs into iTSCs. However, in that study, the time required for transgene expression is considerably longer compared to our study, indicating different conversion kinetics, when Ets2 is absent from the transdifferentiation cocktail [4].

Conventional fetal bovine serum (FBS) containing culture of induced and blastocyst derived trophoblast stem cells relies on the presence of factors secreted by growth-inactivated MEFs [5,6]. During the iTSC induction, these factors are provided by MEFs, which lack the full combination of transgenes and are not undergoing transdifferentiation. However, once individual iTSC colonies are sub-cultured, they require media, which has been preconditioned by growth-inactivated MEFs. From there on, iTSCs can be cultured and treated like blastocyst derived TSCs according to standard protocols. Of note, in contrast to Tanaka *et al.* [5], we routinely culture TSCs and iTSCs without gelatinizing cell culture dishes.

## Protocol

All mouse experiments were conducted according to the German law of animal protection and in agreement with the approval of the local institutional animal care committees (Landesamt fuer Natur, Umwelt und Verbraucherschutz, North Rhine-Westphalia [approval ID number: AZ 84-02.04.2013.A428]).

## 1. Media Preparation

1. Prepare 293T medium: Dulbecco's Modified Eagle Medium (DMEM), 10% (v/v) FBS, L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin/streptomycin (1x).
2. Prepare MEF medium: DMEM, 10% (v/v) FBS, L-glutamine (2 mM), 1x non-essential amino acids, penicillin/streptomycin (1x).

 

3. Prepare TS medium (w/o FGF4/heparin): Roswell Park Memorial Institute medium (RPMI) 1640, 20% (v/v) FBS (stem cell culture grade), penicillin/streptomycin (1x), L-glutamine (2 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (0.1 mM).
4. Prepare TS medium with FGF4/heparin (TS + F4H): RPMI 1640, 20% (v/v) FBS (stem cell culture grade), penicillin/streptomycin (1x), L-glutamine (2 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (0.1 mM), 25 ng/ml FGF4, 1 µg/ml heparin.
   Note: FGF4 and heparin are added directly into the media immediately prior to use.
5. Prepare TS-CM with FGF4/heparin (TS-CM + F4H): 70% MEF-conditioned TS medium + 30% freshly prepared TS medium + 25 ng/ml FGF4, 1 µg/ml heparin.
   Note: FGF4 and heparin are added directly into the media immediately prior to use.
6. Prepare Transfection medium: Advanced DMEM, 2% (v/v) FBS (stem cell culture grade), L-glutamine (2 mM), penicillin/streptomycin (1x).
7. Prepare Virus-production medium: Advanced DMEM, 5% (v/v) FBS (stem cell culture grade), L-glutamine (2 mM), penicillin/streptomycin (1x).

## 2. Murine Embryonic Fibroblast Derivation

1. Set timed matings using wt 129S2SV females and homozygous male R26::rtTA mice (strain name; B6.Cg-Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae/J}$ [7]). On E13.5 sacrifice mice by cervical dislocation and isolate primary murine embryonic fibroblasts (MEFs) according to standard protocols[8]. Thereafter, plate cells from one embryo on a 150 mm dish in MEF media.
2. Once primary MEF cultures reach confluency on 150 mm dishes, wash cells twice with 10 ml PBS and add 4 ml 0.05% trypsin/EDTA solution. Incubate dishes for 3 - 4 min in the incubator at 37 °C.
3. After incubation check if cells detached from the dish using an inverted microscope (using a 10X lens). Add 5 ml of MEF media to stop trypsinization and collect cell suspension into a 50 ml conical tube.
4. Pool cell suspension from several dishes and collect into a 50 ml conical tube. Centrifuge cell suspension for 3 min at 200 x g and 10 °C.
5. Discard supernatant and resuspend cell pellet in FBS containing 10% (v/v) dimethyl sulfoxide. Freeze MEFs in aliquots for further use.
   Note: Alternatively, use wild type primary MEFs; however, an additional lentiviral vector expressing rtTA needs to be transduced together with the other lentiviral vectors (for example using the FUdeltaGW-rtTA plasmid described by Maherali *et al.* [9]).
6. **Preparation of MEF-conditioned TS medium**
   Note: Prior to conversion experiments, MEF-conditioned (CM) TS media has to be prepared, which is required for subculture of individual iTSC lines (described in section 6).
   1. Plate 1 x $10^7$ mitotically inactivated MEFs per 150 mm dish in MEF medium on desired number of dishes. Inactivate MEFs by γ-irradiation with a dose of 9 Gy.
      Note: The yield per 150 mm dish is approximately 60 ml of CM.
   2. The day after plating, aspirate and discard MEF medium and add 20 ml freshly prepared TS medium (without FGF4/heparin) onto each dish of mitotically inactivated MEFs and place dishes back into the incubator at 37 °C.
   3. After incubating TS medium for three days on mitotically inactivated MEFs, collect medium in 50 ml conical tubes. Filter medium through a 0.2 µm filter and proceed with 2.6.4. Replace CM with 20 ml of fresh TS medium for preparation of a further batch of CM.
      Note: Mitotically inactivated MEFs can be used for CM preparation up to three times.
   4. Aliquot 35 ml of filtered CM into 50 ml conical tubes and store at -20 °C until needed. After thawing, add 15 ml of freshly prepared TS medium in order to obtain 70% TS-CM and store at 4 °C for up to one month.

## 3. Lentiviral Vector Production in 293T Cells

Note: All steps are carried out in laboratory space licensed to Biosafety Level 2.

1. Prior to conversion experiments, prepare lentiviral vectors for the doxycycline (dox) dependent over-expression of Tfap2c, Gata3, Eomes, Ets2 and mCherry. For each lentivirus, co-transfect 293T cells using the respective lentiviral transfer vector together with a packaging plasmid and a VSV-G expressing envelope plasmid (for plasmid information refer to the Materials Table).
   Note: For helpful information regarding virus production refer to Gavrilescu *et al.*[10]. Although it describes retrovirus production, general comments regarding quality of 293T cells and plasmid DNA are true for lentivirus production as well.
   CAUTION: Appropriate safety measures need to be taken, when working with lentiviral vectors and work has to be performed in a Biosafety Level 2 facility.
2. Coat five 100 mm dishes with poly-L-lysine (100 µg/ml) solution by covering the dishes with 5 ml of solution and gently rocking to evenly distribute coating solution. Place the dishes back into the incubator at 37 °C for 30 min. After 30 min aspirate coating solution and rinse dishes once with PBS.
3. Plate 7 x $10^6$ 293T cells per dish in 10 ml 293T-cell media, swirl dishes to evenly distribute cells and place dishes back into the incubator at 37 °C.
4. The next morning, replace growth medium with 5 ml of transfection medium. Add 6 µl of a 1,000x stock of chloroquine solution (25 mM) and place dishes back into the incubator at 37 °C.
5. **Meanwhile, prepare transfection mixture:**
   1. For each lentiviral vector prepare two 5 ml reaction tubes, labeled A and B.
   2. Add 600 µl of 2x HEPES buffered saline (HBS) to tube A.
   3. In tube B mix 61.5 µl $CaCl_2$ solution (2 M), with 18.5 µg lentivector DNA and 9.25 µg psPAX2 and 9.25 µg of pMD2.G. Adjust volume to 600 µl with sterile culture grade $H_2O$.
   4. Add solution from tube B drop-wise to tube A while vortexing. Incubate transfection mixture for 15 min at RT.
6. Add transfection mixture very carefully drop wise to the prepared 293T cells on the 100 mm dish. After 5 to 6 hr remove medium and replace with 10 ml of virus-production medium. Place dishes back into the incubator at 37 °C.
   Note: Be careful when removing and replacing medium since 293T cells easily detach from the dish. Medium change 5-6 hr after transfection is crucial, since prolonged exposure to chloroquine is toxic to the cells.
7. The following morning, carefully remove and discard medium and replace with 7 ml of virus-production medium.

8. The following morning harvest the first batch of virus-containing medium. Aspirate medium and collect in a 15 ml conical tube. Again, add 7 ml of virus-production medium onto the cells. Store virus-containing medium in the fridge until the second batch is harvested the following day.
9. The next morning aspirate the second batch of virus-containing medium adding it to the 15 ml conical tube containing the first batch. 293T cells can now be discarded. Filter virus-containing medium through a 0.45 µm surfactant-free cellulose acetate -membrane filter. Aliquot filtered, virus-containing medium and store at -80 °C for further use.
   Note: Virus production can also be performed in 6-well dishes. Scale down volumes and cell numbers accordingly.

## 4. Fibroblast Transduction with 4 Factors and mCherry Control Vector

Note: All steps are carried out in a Biosafety Level 2 facility.

1. For a schematic representation of the experimental outline refer to **Figure 1**. Thaw one aliquot of primary rtTA-MEFs and plate 3.33 x $10^5$ cells per well of a 6-well dish in a total volume of 2 ml of MEF media. Prepare at least 3 wells and label them with 4 factors (4F), mCherry and control.
   Note: Alternatively, wild type MEFs can be used, when an rtTA expressing lentiviral vector is added to the lentivector cocktail during step 4.2.
2. The following day after cells fully recovered from cryopreservation, replace MEF media in the three 6-wells with 0.6 ml (4F), 0.9 ml (mCherry) and 1 ml (control) of Transduction media, respectively. Thaw one aliquot of pLV-tetO-Tfap2c, -Gata3, -Eomes, -Ets and -mCherry virus at RT and add 100 µl of each virus-containing supernatant (Tfap2c, Gata3, Eomes, Ets2) into the 4F well.
   Note: In case of wild type MEFs, reduce volume of preplated media by 100 µl and additionally add 100 µl of FUdeltaGW-rtTA virus containing supernatant to the 4F mix.
3. Add 100 µl of mCherry virus-containing media to the well labeled with mCherry. Add polybrene to a final concentration of 8 µg/ml into each well. Place the dish back into the incubator at 37 °C and incubate MEFs O/N with virus-containing supernatant.
   Note: In case of wild type MEFs, reduce volume of preplated media by 100 µl and additionally add 100 µl of FUdeltaGW-rtTA virus-containing supernatant.
4. The next morning, carefully remove virus-containing media and wash three times with 2 ml of PBS. Finally, add 2 ml of TS media (without FGF4/heparin).
   Note: Transduced MEFs can either be directly used for iTSC induction experiments or frozen for further use.

## 5. Fibroblast to iTSC Conversion

Note: All steps are carried out in laboratory space licensed to Biosafety Level 2.

1. Wash transduced MEFs twice with 2 ml PBS and after adding 0.5 ml trypsin/EDTA solution incubate them in the incubator for 3 - 4 min. Check under an inverted microscope for proper detachment of the cells.
   1. Inactivate trypsin by adding 1 ml TS medium. Transfer the cell suspension into a 15 ml conical tube and centrifuge for 3 min at 200 x g. Discard the supernatant and resuspend the cells in 2 ml TS medium. Use the untransduced control sample to count the cells.
   2. Plate 4F-MEFs, mCherry-MEFs and untransduced control MEFs each at a density of 2 x $10^5$ cells per 100 mm cell culture dish in 10 ml TS medium containing 2 µg/ml dox (TS medium + FGF4/heparin + dox).
   3. Alternatively, perform transdifferentiation on smaller dishes. Plate 3.6 x $10^3$ cells per $cm^2$ on desired dish size, scale volume accordingly.
2. The next day, using an epifluorescence microscope (using a 10X lens and a filter suitable for red fluorescence excitation) monitor expression of mCherry protein in order to estimate transduction efficiency.
   Note: Transduction efficiency should be close to 100%. If increased cell death is apparent after transgene induction this means that virus titer was too high. Transduction should be repeated with reduced amount of virus-containing medium.
3. From now on change media on all dishes every other day until day 10 after plating. Thereafter, feed cells with TS medium without dox (TS medium + FGF4/heparin).
4. Using an inverted microscope monitor for appearance of 'transdifferentiated areas' (refer to **Figure 2B and C**) on 4F dish for up to 3 weeks. Such colonies should not be visible on the two control dishes (mCherry-MEFs and untransduced MEFs).

## 6. Isolation of Individual iTSC Lines

Note: For step 6.3 a tissue culture hood is not required. It is recommended to wipe down the inverted microscope with 70% ethanol to minimize the chances of bacterial contamination. Individual iTSC colonies can be isolated between days 21 and 28 days of transdifferentiation.

1. Add 40 µl of 0.05% trypsin/EDTA solution into 12 wells of a 96 U-bottom well dish and place the dish on ice.
2. Before colony isolation, aspirate medium on 4F-MEF transdifferentiation dish. Wash cells once with 10 ml PBS. Again, add 10 ml PBS and place dish under an inverted microscope.
3. Using a 100-µl pipette set to 40 µl lift individual colonies, by circling them with the pipette tip and aspirating the floating colony. Transfer cells of one colony each into one well of the prepared 96-well dish. After picking 12 colonies place 96-well dish in the incubator at 37 °C for 5 min.
4. Dissociate cells by pipetting up and down for several times and transfer cell suspension into one well of a 24-well dish with prepared 500 µl of TS-CM medium (30% fresh TS medium + 70% CM + FGF4/heparin).
5. The following day replace medium with fresh TS-CM medium to remove dead cells. Change medium every other day.
6. Monitor for appearance of epithelial colonies with bright boundaries (refer to **Figure 2**). Once colonies appear, culture until 70% confluent or for up to one week and gradually expand on larger dishes. From now on, culture cells as bona fide TSCs, according to standard protocols in serum containing media or in serum-free, defined media [3,5,11].

## Representative Results

On the dish where transgene expression of the 4F is activated, cells rapidly change morphology (compare **Figure 2A and B**). Around day 14 - 21 distinct transdifferentiated areas emerge (two examples are given in **Figure 2B and C**). These primary colonies lack typical TSC morphology; however once they are sub-cultured, characteristic epithelial morphology with tight edges and bright boundaries highly reminiscent of bona fide TSCs emerges (**Figure 2D**). 4F-iTSCs stain positive for trophoblast transcription factors Cdx2 and Tfap2c (**Figure 2E**). These iTSC lines can now be used for subsequent *in vitro* or *in vivo* analyses, *i.e.*, *in vitro* differentiation assays or placental chimerization experiments.

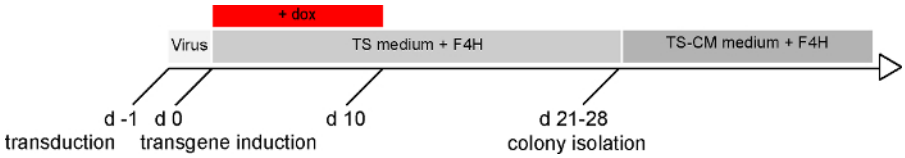


**Figure 1. Timeline of MEF to iTSC Conversion.** Graphical representation of transdifferentiation of MEFs into iTSCs. Please click here to view a larger version of this figure.

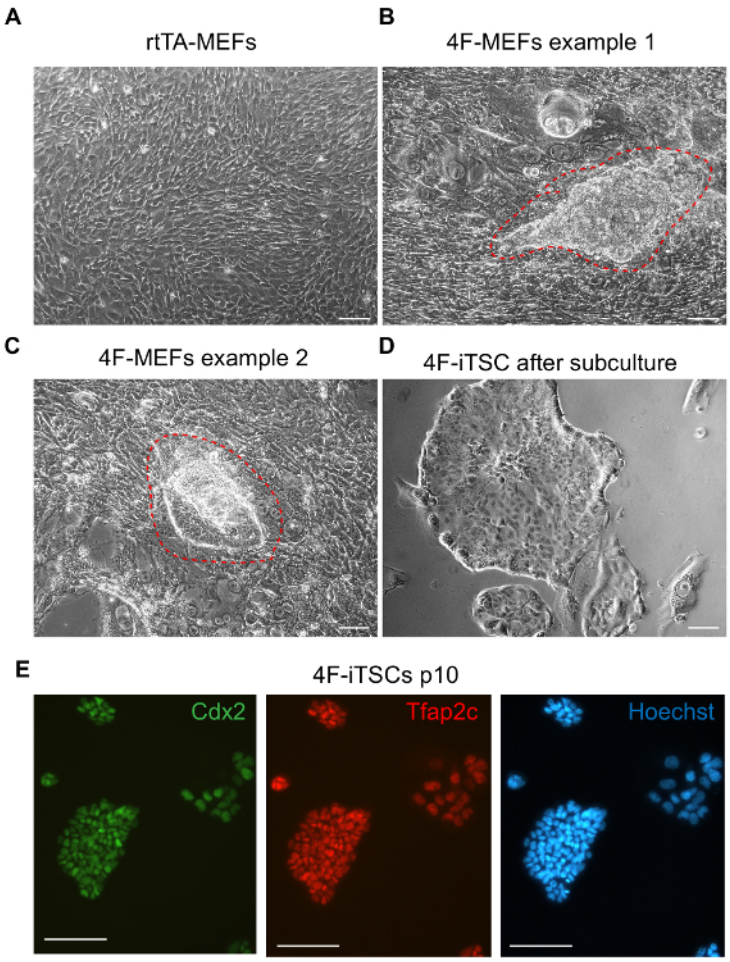


**Figure 2. Representative Changes in Morphology During MEF to iTSC Conversion.**
(**A**) Photomicrograph of rtTA-MEFs. (**B**) Photomicrograph of transdifferentiated 4F-MEFs. Example of a transdifferentiated area, highlighted in red. (**C**) Example 2 of a transdifferentiated colony on day 21 of transdifferentiation after ten days dox induction in 4F-MEFs. Area suitable for sub-culturing highlighted in red. (**D**) Photomicrograph of 4F-iTSCs after sub-culturing. All scale bars indicate 100 µm. Images were taken on an inverted microscope using a 10X lens and phase contrast. (**E**) Immunofluorescence staining against the transcription factors Cdx2 and Tfap2c in 4F-iTSCs. Nuclei are stained with Hoechst. Scale bars indicate 100 µm. Please click here to view a larger version of this figure.

## Discussion

The 4 factor (Tfap2c, Gata3, Eomes, Ets2) based transdifferentiation protocol presented here offers a reliable method to generate faithfully converted iTSCs from mouse embryonic fibroblasts. Further, the method is also applicable for post-natal tail fibroblasts, although with a drop in

efficiency compared to embryonic fibroblasts [3]. In general, quality of primary fibroblasts is a critical factor of transdifferentiation outcome and care should be taken to use early passage cells (passage two to three).

The strength of this protocol is the use of doxycycline-inducible vectors, which allow for temporal control of transgene expression. This enables the generation of stable iTSC lines that activate the endogenous transcription factor network, maintaining TSC fate independent of transgene expression. This is in contrast to previously published protocols, describing the induction of TSC fate from embryonic stem cells, a method that to date only yields incompletely reprogrammed trophoblast stem cell-like cells [1].

However, a known limitation of the protocol described here is the variable number of emerging transdifferentiated colonies, making it difficult to predict the transdifferentiation efficiency. These limitations are due to several highly variable factors, which are critical for the transdifferentiation outcome: the transduction efficiency of the single lentiviral vectors, the amount of proliferation of the starting cell population, and the amount of cell death during transdifferentiation. Under some circumstances transdifferentiated iTSC colonies fail to emerge. In this case media and reagents should be tested for their ability to support genuine TSC culture, before their use in iTSC generation. Additionally, transduction with the 4F can be titrated, since too high amounts of transgene expression lead to cell death. In general, sub-culturing of individual iTSC lines requires some practice. In the event that difficulties with sub-culture of iTSCs occur (*i.e.*, sub-cultured cells fail to maintain self-renewal and instead spontaneously differentiate), single isolated colonies can be alternatively plated on dishes with 50% cell density of growth inactivated feeder cells to support growth and attachment of cells. The iTSC lines can be then gradually weaned from feeders during subsequent passaging. Of note, we did not succeed in directly converting MEFs into iTSCs using defined TX medium conditions, since TX medium only supports established iTSC/TSC lines.

So far, the protocol for iTSC induction from fibroblasts is only established for murine cells. Hence, it will now be of great interest to adapt this protocol to other species and enable derivation of iTSC lines from human or other model systems. Further, analysis of the precise mechanisms of fibroblast to iTSC conversion will offer new insights into the nature of the somatic versus extra-embryonic lineage barrier and aid in understanding principles of cell fate determination during normal and pathological development.

## Disclosures

The authors have nothing to disclose.

## References

1. Cambuli, F., *et al.* Epigenetic memory of the first cell fate decision prevents complete ES cell reprogramming into trophoblast. *Nat commun.* **5**, 5538 (2014).
2. Takahashi, K., & Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell.* **126** (4), 663-676 (2006).
3. Kubaczka, C., *et al.* Direct Induction of Trophoblast Stem Cells from Murine Fibroblasts. *Cell Stem Cell.* **17** (5), 557-568 (2015).
4. Benchetrit, H., *et al.* Extensive Nuclear Reprogramming Underlies Lineage Conversion into Functional Trophoblast Stem-like Cells. *Cell Stem Cell.* **17** (5), 543-556 (2015).
5. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A., & Rossant, J. Promotion of trophoblast stem cell proliferation by FGF4. *Science.* **282** (5396), 2072-2075 (1998).
6. Erlebacher, A., Price, K. A., & Glimcher, L. H. Maintenance of mouse trophoblast stem cell proliferation by TGF-beta/activin. *Dev Biol.* **275** (1), 158-169 (2004).
7. Hochedlinger, K., Yamada, Y., Beard, C., & Jaenisch, R. Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. *Cell.* **121** (3), 465-477 (2005).
8. Jozefczuk, J., Drews, K., & Adjaye, J. Preparation of mouse embryonic fibroblast cells suitable for culturing human embryonic and induced pluripotent stem cells. *J Vis Exp.* (64) (2012).
9. Maherali, N., *et al.* A high-efficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell.* **3** (3), 340-345 (2008).
10. Gavrilescu, L. C., & Van Etten, R. A. Production of replication-defective retrovirus by transient transfection of 293T cells. *J Vis Exp.* (10), e550 (2007).
11. Kubaczka, C., *et al.* Derivation and Maintenance of Murine Trophoblast Stem Cells under Defined Conditions. *Stem Cell Rep.* **2** (2), 232-242 (2014).

 