Animal Husbandry Science PhD School

REPROGRAMMING OF MAMMALIAN SOMATIC CELLS INTO PLURIPOTENT STATE AND THE STUDY OF PLURIPOTENCY

Thesis of the Doctoral (PhD) Dissertation

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1. INTRODUCTION AND GOALS

Pluripotent stem cells are extensively used in various research areas and the practical application of stem cells in clinical therapies has become more realistic. The specialty of these cells is their potential of unlimited division and self-renewal while maintaining their pluripotent state. Furthermore these cells have the potential to differentiate towards any cell types of the three germ layers. Therefore the pluripotent stem cells have important role in basic- and medical-research, drug discovery, disease modelling and therapy.

There are several types of stem cells, of which the most widely used in research are the Embryonic Stem Cells (ESCs). In laboratory conditions the ESCs are isolated from the inner cell mass of blastocyst stage embryos. In case of several species the maintenance of ESC has its limitations and difficulties. Until now there are only a few species where the ESC generation was successful (mice, rat, mammals including human). In addition the maintenance of human ESCs requires the use of human embryos which raises biological difficulties as well as ethical issues. Therefore, pluripotent stem cell maintenance avoiding the use of embryos would markedly advance the stem cell research field. The application of Induced Pluripotent Stem Cells (iPSCs) may overcome some of these issues, as they are generated from somatic cells, with the overexpression of pluripotency factors. The first iPSC was generated by Takahashi and Yamanaka in 2006 where the authors tested several factors and they found the combination of OCT-4, SOX2, KLF4 and C-MYC as the most efficient for reprogramming of somatic cells into pluripotent state. The advantages of iPSCs are that theoretically any type of cells can be reprogrammed and iPSCs carry the donor genotype, thus they can be patient- and/or disease-specific. The iPSCs offer a promising tool in human medicine, such as in disease modelling, drug discovery and therapy. Furthermore the iPSCs may have an important role in the agriculture for transgenic animal production and veterinary applications.

The integrative reprogramming systems are currently among the most efficient methods and still the most commonly applied for basic research. Although in such systems there is a potential risk of insertional mutagenesis, caused by the random integrations what may affect the molecular/functional abilities of the iPSCs if it occurs into an important locus. Non-integrating methods to deliver the reprogramming genes are considered to be the best but they may be inefficient. For basic research the application of integrative but excisable (excisable virus vector, transposon/transposase) reprogramming systems are more feasible, however such strategies are not well studied in the past and require improvement in their excision efficiency. My work described here mainly focused on these systems.

Main goals of the study:

1. Development and optimization of a reliable iPSC reprogramming system on mouse model, by studying integrative (lentivirus, transposon) and non-integrative (protein delivery) methods.

2. Based on the experience gained from the experiments performed on the mouse model, the adaptation of the most efficient reprogramming system(s) to human cells.

3. Full pluripotency characterization of the newly generated iPSC lines following the expansion and long term storage for our research team.

4. Development of a robust reprogramming system, where the integrated transgene(s) can be excised upon reprogramming (excisable lentivirus, transposon/transposase). The work also provided opportunity to study the harmful effect of transgene(s).

5. Future goals: the propagation of transgene-free, disease-specific iPSC lines for ongoing projects in our laboratory, based on the experience gained during this research. As well, the lines generated and characterized during these experiment series will be used as controls.

2. MATERIAL AND METHODS

2.1. Standard methods related to iPSC generation

2.1.1. Cell culture

All cells were cultured at 37°C in humidified atmosphere containing 5% (v/v) CO_2 according to standard cell culturing protocols. In the mouse iPSC experiments bona fide mouse ESCs were used as controls originated from the same genetic background as the iPSCs.

2.1.2. Verification of pluripotency

The pluripotent state of the generated mouse iPSCs was confirmed by the following methods:

- **Gene expression**: RT-PCR was applied for the detection of pluripotency gene expression such as *Oct-4, Sox2, Klf4, c-Myc, Nanog, Rex1, Dax1, FoxD3, Fbxo15,* and *Eras*.
- **Detection of proteins and cell surface markers:** by immunocytochemistry staining (ICC) for OCT-4, NANOG and SSEA-1 markers, then visualized under fluorescent microscope.
- ***In vitro* spontaneous differentiation**: embryoid bodies (EBs) were generated using the Hanging Drop method. On day 2, individual EBs were plated into 1w/24wp and the number of contracting cell clusters was continuously observed and recorded. The clusters were fixed on day 14 and 21, following ICC for differentiation markers (DESMIN, TROPONIN T) visualized under fluorescent microscope.
- ***In vitro* neural differentiation**: EBs were generated by culturing the cells in suspension as previously described (Klincumhom, et al. 2012). Medium was changed every second day; retinoic acid was added (final concentration 5 μM) from day 4. On day 8, the EBs were dissociated with trypsin-EDTA and $2x10^5$cells/cm^2 were seeded into 24-well plate with cover slip for ICC or into 1w/6wp, which were coated with 0.01% poly-L-ornithine and with 1 μg/cm^2 laminin. The cells were fixed on day 10, 12 and 14 of differentiation and ICC was performed for NESTIN and βIII-TUBULIN and then visualized under fluorescent microscope, or NESTIN expression was measured by flow cytometry (FACS).
- ***In vivo* differentiation**: the *in vivo* differentiation potential was determined by chimera test. Production of chimeric animals was done by blastocyst (E3.5) microinjections (PrimeTech, Ibaraki, Japan) where 10-15 cell/embryo were injected (Kawase, et al. 2001). The host embryo was ICR in case of C57BL/6xDBA/2J hybrid and the C57BL/6 iPSCs, while C57BL/6xDBA/2J was in case of ICR-iPSCs. The percentage of live born chimeras was recorded. To test the germ-line transmission ability of the lines, chimeras were mated and the coat colour of born pops indicated the germ-line competency.

The pluripotent state of the generated human iPSCs was confirmed by the following methods:

- **Detection of proteins and cell surface markers:** by immunocytochemistry staining for OCT-4, NANOG, TRA-1-60, TRA-1-81 and E-CADHERIN markers then visualized under fluorescent microscope.

- ***In vitro*** **spontaneous differentiation**: EBs were generated by culturing the cells in suspension. On day 5, 1-3 EBs/cm^2 were plated into 24-well plate. The cell clumps were fixed on day 14 following ICC for three germ layer specific markers (NESTIN, βIII-TUBULIN, GATA4, AFP, BRACHYURY, VIMENTIN) then visualized under fluorescent microscope.

2.2. Generation of mouse iPSCs from three different genetic backgrounds by lentivirus transduction

In the experiments three different mouse strains were used: C57BL/6 (inbred), C57BL/6xDBA/2J (hybrid) and ICR (outbred), hereafter labelled as BL6, F1 and ICR. Mouse Embryonic Fibroblasts (MEFs) were obtained from the three mouse strains and used for reprogramming with our polycistronic excisable lentiviral vector (pF-EF1α/OSKM/IRES/EGFP-W). The vector contained the mouse pluripotency cDNA sequences (*Oct-4-Sox2-Klf4-c-Myc*) and the EGFP reporter under the control of EF1α promoter. In order to make the excision possible using Cre/LoxP system, the LoxP sequence was also introduced into the construct.

For virus transduction, 3x10^4 MEF (0,75x10^4 cell/cm^2) were seeded in *Fibroblast-medium.* Six hours after, the virus-containing supernatant (MOI 2-5) was diluted in 1 ml *mESC-medium* supplemented with 8 μg polybrene and added to the cells. Twenty-four hours after transduction, the medium was refreshed and the cells were cultured one more day. Two days later, the transduced MEF were dissociated with trypsin-EDTA and the cells were plated into 10 cm dish in *mESC-medium*. The medium was refreshed daily. Individual EGFP positive colonies were picked under fluorescence microscope and amplified for further analysis and for determination of EGFP expression level using FACS.

2.3. Generation of lentiviral-mediated transgene-free mouse iPSCs and the verification of transgene excision

The excision ability of the transgene was tested on iPSC lines described above (2.2 section). The transfection of Cre-plasmid (40 μg, pTriEx-HTNC, Addgene, plasmid 13763) was performed by using the Gene Pulser® II Electroporation system according to the user's manual. After electroporation, the cell suspension was seeded into feeder coated 6 cm dish in *mESC-medium*. Two days after transfection, the cells were harvested; counted and 200 cells were seeded onto 0,1% gelatin-coated 10 cm dish. Individual EGFP negative colonies were picked under fluorescence microscope and amplified. The excision was determined by FACS for EGFP.

The transgene-free status of the clones showing no EGFP expression by FACS was also confirmed by transgene-specific PCR reactions (GoTaq® Green Master Mix; Promega, Madison, USA). The presence/absence of transgene was further verified by Southern blot analyses (DIG High Prime DNA Labeling and Detection Starter Kit II; Roche, Basel, Switzerland). The genomic DNA was digested with SphI. The coding sequence of *c-Myc* was used as a probe (1305 bp). As control a mESC was used where only the detection of endogenous *c-Myc* was expected (2,6 kB).

2.4. Generation of mouse iPSCs by protein transduction

For reprogramming, $5x10^4$ ICR-MEFs ($0,5x10^4$ cell/cm^2) were seeded in *Fibroblast-medium*. The cells were subjected to four transduction cycles at 48 hour intervals with the four pluripotency recombinant proteins (8 µg/ml/OCT-4, KLF4, SOX2, C-MYC), as described earlier (Kim et al. 2009; Zhou et al. 2009). On day 9, the cells were gently dissociated with trypsin-EDTA and transferred into 10 cm dishes in *mESC-medium.* The ES-like colonies started to appear around day 40, which were picked and expanded for characterization.

2.5. Generation of lentiviral-mediated transgene-free human iPSCs

2.5.1. Generation of human iPSCs by lentivirus transduction

For reprogramming, Peripheral Blood Mononuclear Cells (PBMCs) were used obtained from five donors. The condition of blood collection and handling was according to „lege artis" regulations, and based on the research permit issued by ETT TUKEB. The blood was collected into ficoll containing 8 ml BD Vacutainer tubes (BD Biosciences, Franklin Lakes, USA). For the isolation of PBMCs the BD tubes were centrifuged and then the separated PBMCs were used freshly or frozen until use. For reprogramming of PBMCs the polycistronic excisable pRRL.PPT.SF.hOKSMco.idTomato.preFRT lentivirus vector was used published by others (Voelkel et al. 2010; Warlich et al. 2011). The vector contained the codon-optimized human pluripotency cDNA sequences (*OCT-4-KLF4-SOX2-C-MYC*) and the dTomato reporter under the control of SFFV promoter. In order to make the excision possible using Flp/FRT system, the FRT sequence was also introduced into the construct.

Three days before virus transduction, $1x10^6$ PBMC ($0,25x10^6$ cell/cm^2) were seeded in *Human PBMC-medium.* Three days later (Day 0. transduction) the cells were transduced with the virus-containing supernatant (1 ml) supplemented with 5 µg polybrene and were plated on 1w/12wp. The plate was centrifuged then incubated overnight. Twenty-four hours after transduction, the medium was refreshed and the cells were cultured one day more. Two days later, the cells were collected plated on feeder coated 6 cm dishes. Thirteen days post-

transduction the medium was changed to *hESC-medium* and refreshed every second days. The appeared hESC-like colonies were individually picked and expanded for further analyses.

2.5.2. The removal of transgene from lentivirus-mediated human iPSC and the verification of transgene excision

The human lentiviral-mediated iPSCs were transfected with the pLV.hCMV-IE.FLPe.IRES.PurR.hHBVPRE plasmid (Gonçalves et al. 2010) using FuGENE-6 transfection reagent (Roche, Basel, Switzerland) according to manufacturer's instructions. 100 µl of FuGENE-6/DNS mix, containing 1 µg Flp-expressing plasmid was added to the cells drop by drop. One day later the cells were selected with 1 µg/ml puromycin for 48 hours.

The transgene-free status of the clones was confirmed by transgene-specific PCR reactions (GoTaq® Green Master Mix; Promega, Madison, USA) and only the transgene-free colonies were picked and expanded individually.

2.6. Generation of human iPSCs by the Sleeping Beauty transposon/transposase system

2.6.1. Generation of human SB-iPSC

The first steps of the experiment were performed in The Netherlands at the LUMC as part of EU project collaboration. Based on the Dutch team's ethical approval Human Foetal Fibroblasts (HFFs) were isolated from an aborted 14-week foetus donated for research after informed consent. For reprogramming the pT2BH-OSKM-IRES/eGFP transposon was used, generated and tested earlier by our research group (Muenthaisong et al. 2012). The transposon plasmid contained the mouse pluripotency cDNA sequences (*Oct-4-Sox2-Klf4-c-Myc*) and the EGFP reporter under the control of the EF1α promoter. $2{,}5x10^5$ HFFs were transfected by nucleofection with 2 µg pT2BH-OSKM-IRES/eGFP transposon and 200 ng pCMV-SB100X transposase, using the Nucleofector kit (Lonza, Basel, Switzerland) according to manufacturer's instruction. The transfected cells were seeded into culture dishes in *Fibroblast-medium,* which was changed to *hESC-medium* 1 day post-transfection and refreshed daily. 7 days after nucleofection, the cells were harvested, seeded on MitC-MEFs, and subsequently cultured in *hESC-medium* until the appearance of hESC-like colonies (~day 26). The appeared ESC-like colonies were individually picked and expanded for further analyses.

2.6.2. The removal of transgene from human SB-iPSC and the verification of transgene excision

The number of transposon integrations was verified by Southern blot analyses (DIG High Prime DNA Labeling and Detection Starter Kit II; Roche, Basel, Switzerland). The genomic DNA was digested with PstI and SacI. The sequence specific for the *EGFP* (Life Technologies,

Carlsbad, USA, 712 bp) or the coding sequence of *c-Myc* was used as probe (1305 bp). The endogenous *C-MYC* was detected by the presence of a 1,5 kB band. The excision of the transgene(s) was performed by the re-transfection of a modified transposase plasmid (pEFBOS-SB100X-iresPuro). The transfection was carried out as it was described before (2.5.2. section). One day later the cells were selected for 48 hours with 1 µg/ml puromycin.

The transgene-free status of the clones was confirmed by transgene-specific PCR reactions (GoTaq® Green Master Mix; Promega, Madison, USA) and only the transgene-free colonies were picked and expanded individually. The genomic location of the transgenes was verified by Splinkerette PCR. In order to further confirm the presence/absence of transgene locus-specific PCRs were performed. The Splinkerette PCR was performed as described earlier (Uren et al. 2009). The genomic DNA was digested with CviQI or DpnII, followed by the ligation of the appropriate splinkerette adaptors. For the Splinkerette PCRs the Platinum Taq high fidelity DNA polymerase was used. TA-cloned PCR products were sequenced bi-directionally with M13 forward and reverse primers, and the sequences analysed using the BLAST search on the NCBI Homo sapiens build 37.3 genome database.

3. RESULTS

3.1. Generation of mouse iPSCs from three different genetic backgrounds by lentivirus transduction

An excisable polycistronic lentiviral vector was designed and used for the reprogramming of somatic cells. The system was highly efficient in the reprogramming of MEF from inbred (BL6), outbred (ICR) and hybrid (F1) mouse strains. Six lines were picked and expanded from each genetic background. The obtained iPSC lines showed pluripotency similar to mESC originated from the same genetic background, based on their morphology and pluripotency marker expression pattern. The success of mESC generation and maintenance depends on the origin of the mouse strain; however we did not observe any impact of the different genetic background on the reprogramming efficiency.

3.2. Generation of lentiviral-mediated transgene-free mouse iPSCs

One of the newly generated F1-iPSC lines was transfected with the Cre-expressing plasmid, which resulted in 13 EGFP negative clones (by FACS analyses) indicating the transgene excision. Based on morphological criteria, two subclones were selected (iPS-Af.4 and iPS-Af.15) and their transgene-free state was further confirmed by transgene-specific PCRs and Southern blot. The morphology of the iPSCs before and after excision was very similar to that of mESC, and expressed all examined pluripotency markers. The effect of the transgenes on the *in vitro* spontaneous and neuronal differentiation ability was assessed by comparing the differentiation of the parental iPSC with two of its transgene-free subclones. We found that the removal of transgene markedly increased the number of contracting clusters during *in vitro* differentiation (iPS-Bef: 39,3%, iPS-Af.4: 66,71%, iPS-Af.15: 54,17%). The excision increased the *in vitro* neural differentiation potential as well, which was assessed by FACS analyses of the NESTIN expression (Day 10 of diff.: iPS-Bef, 10,7±0,3%; iPS-Af.4, 10,7±0,3%; iPS-Af.15, 25,1±0,8%; ESC, 57,1±1,7%). The *in vivo* differentiation ability of the transgene-free lines was also demonstrated by chimera formation, the most stringent test for stem cell pluripotency. Here we showed for the first time that the iPSC lines generated using excisable lentiviral delivery system are germ-line compatible and represents a promising technology to generate transgene-free iPSC.

3.3 Generation of mouse iPSCs by protein transduction

The delivery of the pluripotency factors by repeated transduction of the purified protein cocktail into ICR-MEF resulted in only one iPSC line (piPS-H1). The morphology of the obtained piPS-H1 line was very similar to that of mESC: expressed all examined pluripotency markers and differentiated *in vitro* spontaneously. The piPS-H1 was also able differentiate *in vitro* towards neuronal lineages, where the NESTIN expression assessed by FACS (Day 10 of diff. 49,5%). The piPS-H1 formed chimeras, although the chimeras obtained were not germ-line competent.

3.4 Generation of lentiviral-mediated transgene-free human iPSCs

Using an excisable polycistronic lentiviral vector we generated stable human iPSC from 5 donor's PBMCs. The number of appeared colonies varied among the donors (B#1: ~70, B#2: ~80, B#3: ~40, B#4: ~30, B#5: 1). All maintained iPSCs showed hESC-like morphology, and expressed the pluripotency markers examined. The lines were able to differentiate *in vitro*, confirmed by ICC against lineage specific markers characteristic for the three germ layers.

Seven human iPSC lines were tested for their transgene excision ability. The iPSC lines were transfected with the Flp-expressing plasmid, upon puromycin selection the transgene-free state of the emerged colonies were verified by transgene-specific PCR reactions. In the seven tested iPSCs the excision efficiency of the transgene was as follows: hiPS#1: 98%, hiPS#2: 24%, hiPS#3: 60%, hiPS#4: 20%, hiPS#9: 0%, hiPS#12: 43%, hiPS#13: 40%.

3.5 Generation of human iPSCs by the Sleeping Beauty transposon/transposase system

HFF was transfected with the SB-reprogramming plasmid (previously generated and tested in mouse somatic cell reprogramming by our research team) together with the SB100X transposase vector. Upon transfection four hESC-like colonies emerged generating stable iPSC lines (SB1, SB2, SB4, SB5). All generated iPSCs showed hESC-like morphology, and expressed the examined pluripotency markers. The lines were able to differentiate *in vitro*, which was confirmed by the expression pattern of markers specific for the cells types of the three germ layers.

Based on the Southern blot results, each line contained at least three transposon integrations. Therefore only the SB2 and SB5 lines were selected based on morphological criteria and tested further in excision experiments. By using Splinkerette PCR we were able to determine the insertion location of one the transposon in both cell lines. Based on these results locus-specific PCRs could be also used besides the transgene-specific PCRs in order to monitor the possible excision. Both lines were re-transfected with the SB100X transposase, but following

puromycin selection we could not identify any transgene-free subclones. We also tested whether the repeated transfection may improve the excision efficiency. In case of SB5 with 3 times re-transfection one of the transposon integration excised from its genomic position however it was re-integrated elsewhere along the genome. The excision followed by the re-integration into another site was confirmed by genomic PCRs and Southern blot analyses.

4. NEW SCIENTIFIC RESULTS

1. For the first time in Hungary I have generated and optimized a robust lentiviral-based reprogramming system. The system was sufficient to reprogram mouse embryonic fibroblast into iPSCs, without detecting any influence of the genetic background of the primary cell source. All generated iPSCs showed pluripotency examined by *in vitro* tests.

2. From one of the generated lentivirus-mediated mouse iPSC I was able to excise the transgene, which markedly increased the *in vitro* differentiation potential, compared to parental line carrying the transgene. The transgene-free lines were capable of germ-line competent chimera formation, which was not published before.

3. One transgene-free mouse iPSC line was successfully generated by using protein delivery. The newly generated line performed well both *in vitro* and *in vivo* pluripotency tests.

4. Caused by the re-integration events of the transposons, the excision of the transgene from the newly generated human SB-iPSCs was not possible. While I was able to generate transgene-free lentivirus-mediated human iPSCs using the Flp/FRT recombination system.

5. DISCUSSION AND SUGGESTION

5.1. Generation of mouse iPSCs from three different genetic backgrounds by lentivirus transduction

Previous publications showed that the generation of mouse ESC from 129/SV or C57BL/6 inbred strains is generally the most efficient, while from other inbred/outbred strains might be more difficult (Suzuki et al. 1999; Kawase et al. 1994). Nevertheless, there are only a few publications studying whether the genetic background has any effect on the iPSC generation potential (Schnabel et al. 2012; Muenthaisong et al. 2012). For this reason, the goal of our experiments was to investigate the effect of genetic background on the reprogramming efficiency. Based on our observation we did not find any difference between the three strains examined (BL6, F1, ICR) regarding their reprogramming potential and in the number of emerged colonies. These results confirm our findings gained during the mouse SB-iPSCs generation experiments using somatic cells from three different genetic backgrounds (Muenthaisong et al. 2012). Based on these results we can declare that the integrative iPSC reprogramming systems are highly efficient and offer an alternative way to obtain pluripotent stem cell lines from strains where the ESC generation failed. This might have significance in animal husbandry, for example in generation of transgenic animals where genetically modified stem cells are used. Furthermore the possibility of generating pluripotent stem cell lines from any kind of genetic background has its important role in human disease modelling, because the pluripotent stem cells with different genetic background might show distinct phenotype (Erickson 1996; Sullivan et al. 2007).

5.2. Generation of lentiviral-mediated transgene-free mouse iPSCs

Earlier publication showed already evidences for the negative effect of the integrated transgene on the functional properties of iPSCs (Soldner et al. 2009; Chakraborty et al. 2013). By comparing the differentiation ability of the excised and non-excised lines, provides the opportunity to study the effect of the absence/presence of the transgene on the iPSCs. Even though the number of independent samples was limited, we found that the transgene caused a dramatic effect on the *in vitro* differentiation of the iPSCs. In *in vitro* spontaneous and neural differentiation assays, we found that prior to Cre-excision; iPSC clones had poor differentiation ability, while removal of the transgene augmented the differentiation capacity of the two excised subclones. This observation can be explained with the nature of lentivirus vectors which are often integrated into transcription units (Schröder et al. 2002). Since we did not determine the genomic location of transgenes in the parental iPSC line, we can only assume that the integration occurred into an important locus related to differentiation and caused loss or reduced gene

function. Another possible explanation is that the reprogramming vector contains a promoter which provides stable expression of the exogenous pluripotency genes. Because of continuous, strong expression of the pluripotency factors the iPSCs may not able to differentiate. On the βIII-TUBULIN staining the ectopic expression of EGFP is strong, indicating the presence of active transgenes in our transgene-containing parental iPSC.

Although a large number of studies have provided data on either teratoma formation or chimera mice production as evidence of *in vivo* differentiation capacity of an iPSC line, this does not necessarily mean that the iPSC line is germ-line competent. However the capability of germ-line competent chimera formation is the most stringent criteria of pluripotency and the true measure of differentiation capacity. Our chimera-formation experiments demonstrated for the first time that germ-line competent iPSCs could be generated by a lentiviral gene delivery system once the reprogramming cassette is excisable from the cells.

5.3 Generation of mouse iPSCs by protein transduction

The generation of transgene-free iPSCs using excisable lentivirus system seemed to be highly efficient. Although active transgene sequences do not remained in the genome which would affect the differentiation potential, the excision of transgene leaves a few-hundred bp footprint in the genome, therefore this system could not be considered completely transgene-free. Because of this we also studied a non-integrative system, where the reprogramming was performed by protein transduction. With this method, only one transgene-free mouse iPSC line was obtained. Although the iPSC line performed well in both *in vitro* and *in vivo* pluripotency tests, the formed chimeras were not germ-line competent thus, the extent of reprogramming of these cells is not entirely clear.

The reprogramming efficiency using protein delivery was very low, and labour intensive, in contrast to other integrative systems. Therefore we did not use this system for human iPSC experiments.

5.4. Generation of lentiviral-mediated transgene-free human iPSCs

Here we used a polycistronic excisable lentivirus vector which was published by others (Voelkel et al. 2010; Warlich et al. 2011). With an earlier version of the vector, the authors reported efficient iPSC generation from both mouse and human fibroblasts. It is known that the reprogramming potential of the fibroblasts is one of the highest, however we were able to obtain iPSCs from PBMCs, which cells types has low capability in reprogramming. The major advantage of this cell type over the fibroblast is that it can be obtained in large amount from the donors by non-invasive method. Applying this system we were able to generate lentivirus-

mediated human iPSCs from five donor samples. The number of the emerged hESC-like colonies showed high variation between the donors, which might be caused by their different genetic background.

We attempted the transgene excision from the human lentivirus-mediated iPSCs using the Flp/FRT-recombinase system. In six out of seven lines we were able to obtain transgene-free iPSC lines. Although we used the same system in each case, from one line we were unable to remove the transgene, whereas for the other six lines the excision efficiency showed high variation. These differences could be explained with the random integration of the vector. Presumably, the excision rate differs among genomic locations. An earlier version of the reprogramming vector, containing the non-codon-optimized pluripotency gene sequences was tested for mouse iPSC generation and transgene excision (Voelkel et al. 2010). The authors attempted the removal of transgenes from a mouse iPSC line containing three copies of the transgene by Flp-recombinase protein transduction. From 12 tested subclones none of them showed complete excision of all three integrated transgenes. One of the subclones which only contained 1 further transgene copy was re-transduced with the Flp-recombinase protein. From the eight emerged subclones following transduction, the authors obtained just three transgene-free subclones. In contrast, we observed several transgene-free human iPSCs just after a single transfection of the Flp-expressing plasmid.

5.5. Generation of human iPSCs by the Sleeping Beauty transposon/transposase system

Previous studies have reported that the transposon (PiggyBac: PB) can be used to introduce the reprogramming factors into somatic cells and to generate both mouse and human iPSCs at a similar efficiency to integrative viral-based methods (Kaji et al. 2009; Woltjen et al. 2009; Yusa et al. 2009). The transposon-based system for generating iPSCs also offers several practical advantages over viral-based methods, as its application does not require any advanced biosafety measures that are obligatory for their viral counterparts. Furthermore the transposon vectors are DNA molecules, the generation is a cheaper and simpler method than current viral production protocols, and offers the opportunity to produce xeno-free human iPSCs.

The disadvantage of integrative gene delivery systems is their random integration into the genome with the risk of insertional mutagenesis, however it was shown that the SB transposon does not exhibit an integration bias towards transcriptional units in contrast to lentiviral, retroviral or even PB-transposon system (Mátés et al. 2009; Schröder et al. 2002; Wilson et al. 2007; Wu et al. 2003).

With the set of experiments presented here, we demonstrated that the SB transposon-based system was efficient in generating human iPSCs from HFF. Using this system, just four

iPSCs emerged while the lentiviral delivery system yielded variable number of clones. However the comparison of the reprogramming efficiency of the two different systems is not possible, as the efficiency is influenced by a number of factors such as the primary cell type, cell division potential and genetic background. Besides, the reprogramming efficacy will also depend by the pluripotency factor type carried by the reprogramming cassette as well as from the type of the small molecules applied during the reprogramming process.

Some transposon-based delivery systems offer the possibility to seamlessly excise the reprogramming cassette and potentially generate genetically unmodified iPSCs by the re-activation of transposase enzyme. While this was achieved in mouse iPSCs using PB-transposon as a proof-of-principle (Woltjen et al. 2009; Yusa et al. 2009), no similar human iPSC lines have been reported. To attempt this we re-transfected our human SB-iPSC lines with the SB100X transposase vector, however the excision of the transgenes was failed. Although we were unable to generate transgene-free iPSCs, we did detect the mobilization of the integrated transposons in the SB5 after repeated transfections, where one of the transposon integration was excised but re-integrated elsewhere in the genome. Our results suggest that the transposon/transposase system in its current state is not efficient in generating transgene-free iPSCs.

Major conclusions based on our results:

The presence of transgene may impact the differentiation potential; therefore even for *in vitro* research applications removal of the transgene is highly desirable.

The results indicate that due to the different nature of the vectors, the removal of the recombinase system-based excisable lentivirus vectors work with higher efficiency, in reproducible, and in species independent manner. Based on these the data, the use of excisable lentiviral-mediated systems for transgene-free iPSCs generation is suggested.

Upon the excision of lentiviral vector, a few-hundred bp footprint still remains in the genome, which does not contain active transgene sequences, however the lines cannot be considered completely free of genomic manipulations. Therefore the application of such system are more reliable for laboratory research purposes.

6. PUBLICATIONS

6.1. International and Hungarian publications in the subject of the dissertation

6.1.1. International journals with impact factor

Dambrot, C., Buermans, H.P.J., **Varga, E**., Kosmidis, G., Langenberg, K., Casini, S., Elliott, D.A., Dinnyes, A., Atsma, D., Mummery, C., Braam, S., Davis, R.P Strategies to rapidly map proviral integration sites and assess cardiogenic potential of nascent human induced pluripotent stem cell clones. Exp Cell Res. 2014 Oct 1;327(2):297-306. IF: 3,557

Varga, E., Nemes C., Davis, R.D., Ujhelly, O., Klincumhom, N., Polgar, Z., Muenthaisong, S., Pirity, M.K., Dinnyes, A. Generation of transgene-free mouse induced pluripotent stem cells using an excisable lentiviral system. Experimental Cell Research, 2014 Apr 1;322(2):335-44. IF: 3,557

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6.1.2. International Poster/Abstract/Oral presentation/Conference publications

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Chandrasekaran A, Neelchen Roesingh L, Ochalek A, Nemes C, **Varga E**, Bock I, Szczesna K, Avci H, Kobolak J, Dinnyes A. Comparison of 2D and 3D neuronal differentiation of patient specific induced pluripotent stem cells XV. Biannual Conference of the Hungarian Neuroscience Society, Budapest, Hungary 22-23 Jan. 2015. Abstract and Poster

Tancos, Zs., Ochalek A., Bock I., **Varga E**., Nemes Cs., Dinnyes A. Generation of rabbit induced pluripotent stem cells (rbiPSCs) by human reprogramming factors. Salaam Opening Conference. Munich, Germany. 15. Dec. 2014. Abstract (p38) and Poster

Chandrasekaran A., Neelchen Roesingh L., Ochalek A., Nemes C., **Varga E**., Bock I., Avci H., Kobolak J., Dinnyes A. Neuronal differentiation of patient specific induced pluripotent stem cells. EMBO Conference Stem cells in Cancer and Regenerative Medicine, Heidelberg, Germany 9–12 Oct. 2014. Abstract and Poster

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6.1.3. Hungarian Poster/Abstract/Oral presentation/Conference publications

Tancos, Z., Ochalek A., Nemes C., **Varga E**., Bock I., Dinnyes A. Generation of rabbit induced pluripotent stem cells (iPSCs) by human reprogramming factors. in Fiatal Biotechnológusok Országos Konferenciája (FIBOK 2014). Szeged, Hungary. 7. March 2014. Abstract and Poster (p.86)

Varga, E. Transzgén-mentes Indukálható Pluripotens Őssejt vonalak alapítása Egér modellben. (Maintenance of Transgene-free mouse iPSCs). Állattenyésztés-tudományi Doktori Iskola fórum. Szent István Egyetem, Godollo, Hungary, 19. Jun. 2013. Abstract

Dinnyes, A., **Varga, E**., Berzsenyi, S. Betegspecifikus őssejtek, majd idegsejtek előállítása bőr és vér minták genetikai újraprogramozásával. (Generation of diseased-specific iPSCs and neurons from skin and blood primary cell sources). Magyar Tudományos Parkinson Társaság konferenciája. Budapest, Hungary, 31. May - 1. June 2013. Abstract (p. 1-3.) and Presentation

Dinnyes, A., Berzsenyi, S., Nemes, C., **Varga, E**., Kobolak, J. Emberi indukált pluripotens őssejtek az idegrendszeri és más örökletes betegségek kutatásának szolgálatában. Transzlációs klinikai idegtudományok: az omikától a proteomikáig. (Human iPSCs for neurodegenerative and other familiar disease research). Velence, Hungary, 6-7. Dec. 2013. Abstarct

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6.2. International and Hungarian publications non-related to the subject of dissertation

6.2.1. Hungarian journals with impact factor

Varga, E., Polgar, Z., Bodo, S., Dinnyes, A. Lézer asszisztált in vitro fertilizáció fagyasztott spermával nyúl modellben. Magyar Állatorvosok Lapja, 2009. 131(9): p. 562-565 (Laser-assisted in vitro fertilization with frozen rabbit semen in rabbit model) IF: 0,146

6.2.2. Hungarian journals without impact factor

Varga, N., **Varga, E.**, Almassy, Z. A Hunter-szindróma korai felismerésének lehetőségei és diagnosztikai lépései. Gyermekorvos Továbbképzés. 2012.11. évfolyam 5. Szám (Possibilities and diagnostic steps for early detection of Hunter syndrome)

6.2.3. International Poster/Abstract/Oral presentation/Conference publications

Zhou S., Szczesna K., Avci H., Kobolák J., **Varga E**., Schmid B., Ochalek A., Rasmussen M., Freude K., Cirera S., Dinnyés A., Hyttel P. Role of bFGF and EGF in neural rosette formation, ISSCR 2015, Stockholm, Sweden, 24-27. June 2015. Abstract

Polgar, Z., Boonkusol, D., **Varga, E**., Dinnyes, A. In vitro development of vitrified in vivo and in vitro fertilized pronuclear-stage rabbit embryos. Reproduction in Domestic Animals, ESDAR, 2010. 45(3). Abstract (p. 69.) and Poster

Polgar, Z., Boonkusol, D., **Varga, E**., Dinnyes, A. Efficient vitrification of pronuclear-stage rabbit embryos. in Proceeding of the 3rd General Meeting of GEMINI. Soustons, France, 1-3. Oct. 2010. Abstract (p. 60.)

Varga, E., Polgar, Z., Bodo, S., Dinnyes, A. Increase of fertilization with frozen semen in laser-assisted rabbit in vitro fertilization. Magyar Allatorvosok Lapja, 2009. 131(9): p. 562-565. Abstract and Presentation

Varga, E., Polgar, Z., Bodo, S., Dinnyes, A. Laser-assisted zona-drilling increased in vitro fertilization with frozen semen in rabbit. Reproduction in Domestic Animals, ICAR 2008, Budapest, Hungary, 13-17. Jul. 2008. Abstract (43: p. 138-139) and Poster (#337)

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