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Animal Husbandry Science PhD School

ISOLATION AND IN VITRO ANALYSIS OF PLURIPOTENCY GENES IN RABBIT

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1. INTRODUCTION AND GOALS

One of the goal of the current work was the identification of rabbit *OCT4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, *FGF-4*, *UTF-1*, *REX-1* and *FBX-15* transcription factor genes which play an important role in forming pluripotency by isolating the whole coding regions or the partial mRNA sequences. The embryogenesis of mammalian embryos and the pluripotency of the cells are coordinated by several genes. Genes examinated in this study are also included in this goup. They are coding transcription factors which play an important role in actuation of production of other proteins and in coordinating the formation of germ line. At molecular level the toti- and pluripotency cannot be connected clearly to any of the pluripotency factors. Nowadays we think, that the state of pluripotency can be described as a result of a cooperation of several genes.

The sequences of known pluripotency genes were analyzed and primers designed based on the similarity of sequences. Sequences of each individual rabbit pluripotency gene was compared to other vertebrates (e.g. human, mouse, bovine) phylogenetically. Rabbit ESCs and blastocyst stage embryos were collected from superovulated rabbits to isolate total RNA. Genes of interest were amplified using RT-PCR and electrophoretically separated for cDNA fragment isolation. Isolated and subcloned cDNA fragments were sequenced and analyzed. Based on the identified sequences species- and genespecific primers have been designed for gene isolation. For the examination of the function of identified genes the one of the most suitable system is the pluripotent cell analysis. Therefore our experiments were performed on rabbit pluripotent (ES and iPS) cells. We think with the application of species-specific reprogramming factors more stable rabbit iPS lines could be generated, thus our goal is the generation of rabbit induced pluripotent stem cell lines and isolation of genes which are indispensible for these experiments.

Rabbit is one of the most relevant and frequently used non-rodent species for animal models. The main biomedical applications of GMO (Genetically Modified Organism) rabbits are biopharming (live bioreactors) for large scale production of pharmaceutically important proteins for the treatment of human diseases, and models for human diseases.

Our additional goal is the generation of rabbit induced pluripotent stem cell lines using human and mouse reprogramming factors which could be resulted in a comparative system where the differences and similarities between different species can be studied.

Objectives of this study

1. Detection of pluripotency marker genes (*OCT4, NANOG, SOX-2, C-MYC, KLF4, FGF-4, UTF-1, REX-1* AND *FBX-15)* in rabbit pre-implantation stage embryos, using primers designing for consensus sequences.

2. Production of species-specific primers and elaboration of species-specific RT-PCR reactions in order to to detect expression of pluripotency marker genes in rabbit.

3. Generation of rabbit induced pluripotent stem (iPS) cells using mouse and human genes as reprogramming factors by Sleeping Beuty transposon system.

4. Generation of rabbit iPS cells using mouse and human genes as reprogramming factors by Lentiviral transposon system.

5. Creation of reprogramming vector using our newly isolated rabbit pluripotency genes in order to generate rabbit IPS cells.

2. MATERIAL AND METHODS

2.1 Superovulation of donor animals

For oocyte collection Hycole hybrid female rabbits (Charles River, Sulzfeld, Germany) were used, with similar body composition to New Zealand White rabbits. These animals were induced to superovulate by intramuscular (i.m.) administration of 120 IU (international units) of pregnant mare serum gonadotropin (PMSG, Folligon Intervet, The Netherlands) followed 72 h later by intravenous (i.v.) injection with 170 IU human chorionic gonadotropin (hCG, Choragon Richter Gedeon Rt., Hungary).

2.2 In vivo embryo production

For collection of in vivo derived embryos, superovulated and fertilized donor females were sacrificed at various time intervals post hCG administration, and 2-cell- (26 h), 4-cell- (35 h), 8-cell- (45 h) embryos and blastocysts (98 h) were individually collected. During sample collection, after the removal of the zona pellucida with Pronase (Sigma-Aldrich, St Louis, MO, USA) the denuded oocyte and preimplantation embryos were washed three times with Phosphate-Buffered Saline (PBS) supplemented with 20% Fetal Bovine Serum (FBS) and pooled (10 embryos/ pool) in 2 μl RNase-free water and stored at −80 °C until RNA extraction.

2.3 In vivo oocyte collection

Donor females were sacrificed at 16 h post hCG administration and the oocytes were flushed from the ampullae of the oviducts with M2 medium (Sigma-Aldrich, St Louis, MO, USA). First oocytes were freed from cumulus cells using 0.1% Hyaluronidase in M2 medium. Then the zona pellucida was removed using 0.5% Pronase (Sigma-Aldrich, St Louis, MO, USA). The denuded metaphase 2 oocytes (con fi rmed by the presence of a single polar body) were collected into pools (10 oocytes/ pool) for RNA isolation.

2.4 In vivo tissue collection

Adult Hycole hybrid female rabbits were euthanized and liver, heart, kidney, brain, spleen, stomach, ovary, and lung tissues harvested. Tissues were dissected into pieces of approximately 25 mg wet weight using a scalpel, then frozen on dry ice and stored at − 80 °C for RNA extraction.

2.5 Cell culture

Cell culture media and supplements were purchased from Life Technologies (Carlsbad, CA, USA) unless otherwise specified. Rabbit embryonic stem cells (rESC) used in this study (kindly provided by Professor A. Honda, RIKEN Laboratory, Japan) were cultured as described previously (Honda et al., 2008). Briefly: rESCs were treated with 0.05% Trypsin/EDTA for 1 min at room temperature (RT). The enzyme was stopped with iPSM medium (78% DMEM/Ham's/F-12 supplemented with 20% knock-out serum replacement, 2 mM GlutaMax, 1% nonessential amino acids (Sigma Aldrich), 0.1 mM β-mercaptoethanol, 10 3 units/ml ESGRO (murine LIF; Millipore, Bedford, MA, USA),and 4 ng/ml human recombinant basic fibroblast growth factor). Cells were passaged every 3–4 days. For RNA extraction, the cells were washed with PBS and stored at −80 °C.

2.6. Primer design

Primers used for isolation of full-length coding regions of rabbit pluripotency-associated genes were designed to anneal to highly conserved regions of the genes using Primer3 software (Rozen and Skaletsky, 2000). The conserved regions of the orthologous sequences of each pluripotency-associated gene studied were determined by aligning the sequences of the orthologous genes from the human, mouse, bovine and cat using ClustalW software (http://www.ebi.ac.uk/ Tools/msa/clustalw2/)

2.7 Cloning and sequencing of POU5F1, SOX2, KLF4, C-MYC and NANOG

mRNA cDNA synthesized from blastocyst RNA was subjected to PCR. The amplification reactions were carried out in a total volume of 25 µl, containing 10 embryo equivalent cDNA, 0.2 µM of each primer, and proofreading enzymes, either Premix Ex Taq (TAKARA Bio, Otsu, Japan) or Phusion Hot Start II High- fidelity DNA polymerase (Thermo Scientific, Waltham, MA USA). The isolated fragments were cloned into target vectors using the TOPO TA Cloning or the Zero Blunt TOPO kits (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Sanger sequencing reactions were performed on the cloned PCR-products using an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.10 Sequence analysis and comparative genomics

Quality control and assembly of the sequence reads were performed by CLC Main Workbench 5 software (CLC Bio, Aarhus, Denmark). ApE software (M. Wayne Davis) was used to screen for the open reading frames of the assembled sequences. We were able to detect coding regions (CDS) and predict protein products for all of the studied genes. To assess the accuracy

of the automated computational analysis (Gnomon, Souvorov et al., 2010) performed for rabbit SOX2, KLF4, C-MYC and NANOG, the newly determined protein products were aligned to the predicted rabbit protein sequences retrieved from the GenBank Database (SOX2: XP_002716497.1, KLF4: XP_008253622.1, C-MYC: XP_002710590.1, NANOG: XP_002712808.1) using EMBOSS Needle software (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The POU5F1 protein sequence described by Shi et al. (2008) (GenBank accession number: NP_001093427.1) was aligned to the sequence identified in this study. For detailed evolutionary conservation analysis, first the coding regions of the studied genes were aligned to their human, mouse, bovine and cat counterparts (using EMBOSS Needle software (http:// www.ebi.ac.uk/Tools/psa/emboss_needle/). Then, protein sequences from these species were compared to the rabbit protein sequence. The conserved domains of the rabbit proteins were identified using EMBOSS Water software (http://www.ebi.ac.uk/Tools/psa/emboss_water/).

2.10 Creation of Sleeping Beauty plasmid constructions

Sleeping Beauty (SB) transposon was provided by Ivics and his collegues (Ivics et al. 1997). For our experiments we used this construction for creation of our polycistronic vectors.

2.11 Reprogramming vector containing mouse plurypotency genes

The mouse OSKM fragment from the FUW-OSKM construct (Carey et al. 2009) (Addgene plasmid 20328) was cut at EcoRI sites, and then inserted into the SB construct at the EcoRI site (Muenthaisong et al. 2012)

2.12 Reprogramming vector containing human plurypotency genes

The human OKSM fragment was cut from the pRRL.PPT.SF.hOKSMco.idTomato.preFRT construct and then inserted into SB construct using Gibson Assembly reaction (Gibson *et al.* 2009).

2.13 Reprogramming vector containing rabbit plurypotency genes

The rabbit OSKM casette was created using our isolated rabbit pluripotency genes then inserted into SB construct using Gibson Assembly reaction (Gibson *et al.* 2009).

2.14 Gibson Assembly cloning method

Gibson Assembly efficiently joins multiple overlapping DNA fragments in a single-tube isothermal reaction. In case of SB vector the human, mouse and rabbit reprogramming factors and linearized SB vector were joined to each other using this method.

2.15 Lentiviral plasmid constructions

2.15.1 Lentiviral vector containing mouse and human reprogramming casette

For introduction of mouse reprogramming factors the pF-EF1-α/OSKM/IRES/EGFP-W construct was used (Varga et al. 2014).

For introduction of human reprogramming factors the pRRL.PPT.SF.hOKSMco.idTomato.preFRT construct was used (Varga et al. 2014).

2.16 Culturing of rabbit hepatocyte, fibroblast and HEK293T cells

The cells was cultured in T75 flask at 37 °C under 5% CO2 in air.

2.17 Culturing of rabbit ES and IPS cells

Rabbit embryonic stem cells (rESC) used in this study (kindly provided by Professor A. Honda, RIKEN Laboratory, Japan) and rabbit IPS cells were cultured as described previously (Honda et al., 2010).

2.18 Generation and culture of rabbit iPS cells

2.18.1 Lipofection

REFs were seeded onto 24-well plates at a density 0,5-2 × 10^5 cells/well one day prior to transfection in FM medium without antibiotics. The next day (day 0), 4 μg of pT2BHEF1α-OSKM-IRES/eGFP expression vector and 0.4 μg of transposase (SB100X (Mates et al. 2009)) were co-transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. On day 2, transfected REFs were trypsinized with 0.25% trypsin, 0.1% EDTA and re-plated onto 10 cm tissue culture dishes containing mitoMYCin-C treated MEFs

2.18.2 Lentiviral transduction

REFs were seeded onto 24-well plates at a density 3 × 10^4 cells/well one day prior to transfection. The next day polybrane was added with 8 μg/ml final concentration, then the virus was added as well using several differents virus/ cell number proportion: 0.5, 1, 2 és 4.5 MOI (multiplicity of infection)

2.19 Alkaline phosphatase staining and immuncitochemistry

To detect alkaline phosphatase activity, rabbit iPS cells were stained using an alkaline phosphatase kit (Sigma-Aldrich, Saint Louis, MO, USA) and AP live stain kit (Life Technologies) according to the manufacturer's protocol. Quantitative analysis of relative activity of alkaline phosphatase was assessed using OLYMPUS IX71 fluorescence microscope.

For immuncitochemistry the cells were permeabilized in PBS containing 0.1% Triton X-100 for 5 min and blocked by 1% bovine serum albumin for 1 h at RT. Cells were then incubated overnight at 4 °C with primary antibodies, then the primary antibodies were visualized with AlexaFluor 488 donkey anti-goat IgG (H + L), AlexaFluor 488 donkey anti-mouse IgG (H + L) (Life Technologies, California, USA, diluted 1/2000). Finally, nuclei were counterstained with DAPI (4′ ,6-diamidino-2-phenylindole; 0.2 μg/ml fi nal concentration) in PBS for 20 min at RT. Cells were examined using a fluorescence microscope equipped with a 3D imaging module (AxioImager system with ApoTome, Carl Zeiss MicroImaging GmbH, Jena, Germany) controlled by Axio-Vision 4.8.1 microscope software.

2.20 Differentiation *in vitro* and *in vivo*

To test for embryoid body (EB) formation, iPS cells were digested with 0.1% trypsin, resuspended in a solution containing 78% DMEM/Ham's F-12, 20% KSR, 2 mM GlutaMax, 1% nonessential amino acids, and 0.1 mM ß-mercaptoethanol, and cultured in Petri dishes. EBs were collected after 4–7 days in suspension culture and transferred to plastic dishes coated with gelatin to promote adherence. Culture continued for an additional 14–21 days to promote further differentiation. For endodermal differentiation, rabbit iPS cells were cultured using 78% DMEM/Ham's F-12, 20% KSR, 2 mM GlutaMax, 1% nonessential amino acids, 0.1 mM ß-mercaptoethanol, 4 ng/ml bFGF, and 50 ng/ml activin A (R&D systems, Minneapolis, MN, USA) on feeder free gelatin-coated dishes for 5 days. The outgrowths were then fixed with 4% paraformaldehyde for 30 min at room temperature and then washed three times (5 min each) with 1% bovine serum albumin (BSA) in wash buffer. For permeabilization, cells were treated with 0.1% Triton X-100 in wash buffer for 10 min. Cells were incubated with blocking solution (10% normal donkey serum and 1% bovine serum albumin in wash buffer) for 30 min. The following primary antibodies were used: anti-beta III tubulin, anti-GFAP (R&D systems), anti-alpha smooth muscle actin (Abcam, Cambridge, MA, USA), and anti-GATA4 (Santa Cruz Biotechnology). All antibodies were diluted in blocking solution and incubated with samples overnight at 4 °C. The next day, cells were washed three times with wash buffer and incubated with secondary antibodies at room temperature for 60 min. Cells were washed three times with wash buffer and covered with 50% glycerol containing DAPI. The fluorescent signals were

analyzed using an OLYMPUS IX71 fluorescence microscope.

For teratoma formation, 1–2 x 10^6 iPS cells were injected under the kidney capsule of five- to eight-week-old SCID mice. After four to eight weeks, teratomas were dissected and fixed in paraformaldehyde as above. Paraffin wax sections were stained with hematoxylin and eosin

3. RESULTS

3.1 Identification of rabbit pluripotency genes

The embryogenesis of mammalian embryos and the pluripotency of the cells are coordinated by several genes. The proteins and the gene products which are coding them are detected not only in the pre-implantation stage embryos, but also in the embryonic stem cells.

Embryonic stem cell lines can be used as an *in vitro* model of the cell differentiation process which we can study by the isolation of the genes we detected.

3.2 Main factors for selection, analysis and generation of pluripotent cells

The goal of the our rabbit iPS work was to close the gap in knowledge with regard to the molecular biological background for rabbit iPS work by isolating the putative pluripotency genes from the rabbit, based on the sequences published for other species. The sequence of known pluripotency genes (*OCT4, SOX2, KLF4, C-MYC, NANOG*) were analysed and primers designed based on the similarity of sequences. Sequences of each individual rabbit pluripotency gene was compared to other vertebrates (e.g. human, mouse, bovine) phylogenetically. Rabbit ESCs and blastocyst stage embryos were collected from superovulated rabbits to isolate total RNA. Genes of interest were amplified using RT-PCR and electrophoretically separated for cDNA fragment isolation.

In conclusion, we have revealed differences at both Na and Aa level in all four major rabbit pluripotency gene sequences in comparison to their mammalian orthologs which might partially explain difficulties in generation of rabbit iPSCs capable of germline transmission.

Based on the high conservation observed for the rabbit pluripotency-associated genes we speculated they may have a similar role as their human orthologs during the embryogenesis. For this, we examined the expression patterns of the newly identified genes during rabbit preimplantation development by RT-PCR. All the five investigated genes were determined to be highly expressed during pre-implantation development. Particularly, *OCT4* and *KLF4* express constantly in oocytes and during the early development, while *NANOG* is activated only after fertilization. *C-MYC* and *SOX2* are transcribed from the 8 cell stage that is maintained in the blastocysts. Interestingly, we also observed a minor activation of *C-MYC* at the 2 cell stage. Next, the specific expression patterns of the pluripotency-associated genes were determined in different adult rabbit tissues and in rabbit embryonic stem cells as a positive control (Honda et al. 2008). We found varying levels of expression of all the five investigated genes in most of the rabbit tissues. Particularly, *OCT4* was expressed in all tissues excluding the lung and showed

very low expression level in the liver. *SOX2* and *C-MYC* were expressed throughout. *NANOG* expression was lacking in the brain and *KLF4* expression in kidney and ovary.

Our results clearly demonstrate that the studied rabbit pluripotency-associated genes are not restricted to the embryonic development. Based on the gene expression data, it can be assumed that the studied genes possess conserved roles in the maintenance and renewal of different organs of the rabbit.

3.3 Other factors for selection and analysis of pluripotent cells

In case of *UTF1, REX1* and *FBX15* genes, the goal of this work was to isolate these genes from rabbit, based on the sequences of other species published so far. The sequence of known genes has been analyzed, and primers have been designed based on similarity of sequences. Oocyte-to-blastocyt-stage embryos were collected from superovulated rabbits.

The expression of these genes was analysed by RT-PCR. At this point, it can be stated that, that all of these genes have been successfully identified in rabbit genome, a method has been developed to detect their expression and these genes are suitable for detecting of endogen genes in generated induced pluripotent cell lines.

3.4 Generation of rabbit iPS cells

Honda et al. were the first group to report the establishment of rbiPSC lines which they produced from adult rabbit liver and stomach cells using lentiviral vectors carrying the human reprogramming genes: *C-MYC, KLF4, SOX2* and *OCT4.* The newly generated rbiPSCs resembled human iPSCs; they formed flattened colonies with sharp edges and proliferated indefinitely in the presence of FGF2. They expressed the endogenous pluripotency markers *C-MYC, KLF4, SOX2, OCT4* and *NANOG*, while the exogenous human genes were completely silenced. *In vitro*, rbiPSCs rapidly differentiated into ectoderm, mesoderm and endoderm. *In vivo* they formed teratomas, but they were unable to form chimeras.

Our goal was to verify that applification of rabbit specific pluripotency genes to reprogram somatic cells and generate iPSCs might be more efficiently than by using mouse or human genes.

4. NEW SCIENTIFIC RESULTS

1. Coding region of rabbit *SOX2, NANOG, KLF4, C-MYC* pluripotency markers was identified succesfully and submitted in the GenBank database with the following accession numbers: KJ939365, KM267140, KJ708483, KJ708482.

2. For the first time, I have identified the whole coding region of rabbit *SOX2, NANOG, KLF4, C-MYC* pluripotency markers in rabbit preimplantation stage embryos using primers designed on the consensus sequences. These genes might be able to reprogram rabbit somatic cells.

3. I have identified succesfully the partial mRNA sequences of *FGF4, UTF1, REX1, FBX15* rabbit pluriponcy markers using primers designed on the consensus sequences. With these genes we can able to detect the endogenous gene expression in rabbit induced pluripotent stem cells

4. I have designed species-specific primers and developped species-specific RT-PCR reactions for detection of *OCT4, SOX2, NANOG, KLF4, C-MYC, FGF4, FBX15, UTF1* and *REX1*. Using these RT-PCR reactions, I have succesfully annalysed the expression of *OCT4, SOX2, NANOG, KLF4, C-MYC* genes in preimplantation stage embryos and in adult tissues.

5. Introduduction of mouse and human pluripotency genes using a polycistronic vector, for the first time I have generated rabbit iPS cells by transposon (Sleeping Beauty) and lentiviral system as well. The generated cell lines was characterized and their properties were compared to pluripotent ES cells.

6. Species-specific reprogramming vector was created using the identified rabbit pluripotency factor genes

5. DISCUSSION AND SUGGESTION

5.1 Identification of rabbit pluripotency genes

We were able to amplify the coding sequences of rabbit *OCT4, SOX2, KLF4, MYC* and *NANOG* genes using PCR primers binding conserved regions of the targeted genes. These genes were submitted in NCBI database with the following accession numbers: *OCT4*: KJ708484, *SOX2*: KJ939365, *KLF4*: KM267140, C-*MYC*: KJ708483, *NANOG*: KJ708482. The protein sequence of the OCT4 gene that was already determined by Shi et al. (2008) was used as a reference. Interestingly, 11 (3%) amino acid differences are present between the sequence of Shi et al. (2008) and ours. We detected 18 amino acid (aa) differences by aligning the predicted (GeneBank data) and the determined SOX2 protein sequences. Interestingly, the Gnomon computational analysis indicated a 9 aa long glycine repeat at the N-terminus of the SOX2 protein that was not present in our sequence. We found perfect homology between the overlapping regions of the predicted and our determined MYC and NANOG sequences however, in case of the NANOG there was a difference between the length of the predicted and experimentally determined sequences. We detected a stop codon (TGA) 50 aa earlier than the computational analysis did. In addition, we are the first to report the complete coding sequence of the rabbit KLF4 as only fragmented sequence prediction was available for this gene (GenBank). The alignment of the predicted and experimentally determined *KLF4* sequences showed perfect homology at their N- and C-terminus, however there was a >200 aa gap missing from the prediction.

Furthermore, the degree of evolutionary conservation of the newly identified genes was evaluated by phylogenetic analysis. The comparison of the rabbit coding and protein sequences to the human, mouse and bovine orthologs revealed that the SOX2 gene is highly conserved with only 0.6−2.8% differences between the protein sequences of the investigated species. There are only 6 nucleotides and 2 aa differences between the rabbit and mouse sequences, which indicates almost perfect identity. Furthermore, the sequences of OCT4, KLF4 and MYC are also evolutionary well preserved, while NANOG differs significantly both at nucleic and amino acid levels partially due to the anterior stop codon present only in rabbit. Regarding the coding sequences the cat showed the highest homology to the rabbit for 3 out of 5 mRNAs. Nevertheless, this was only partially reflected in the protein level as rabbit KLF4 was more similar to the bovine sequence. Additionally, the human and the cat protein sequence show equal similarity to the rabbit OCT4. Importantly, in case of the NANOG the smallest difference was observed between the human and rabbit sequences. The rabbit MYC protein is evolutionary closer to its cat and bovine counterparts, as they lack a 15 aa long overhang that is present in the

N-terminus of the human and mouse protein products. To investigate the function of the newly identified rabbit genes we screened them for conserved domains of the human proteins. All the domains that are present in the human genes (according to GeneBank) were apparent in rabbit sequences and showed strong conservation. Interestingly, the human and rabbit Zinc-finger double domains of the KLF4 perfectly (100%) match. Additionally, almost complete homology was observed for SOX2 SOXp and SOX-TCF HMG-box domains and for the MYC Helix-loop-helix domain. The least, but still highly conserved sequence was the NANOG homeodomain with 91.4% identity.

Based on the high conservation observed for the rabbit pluripotency-associated genes we speculated they may have a similar role as their human orthologs during the embryogenesis. For this, we examined the expression patterns of the newly identified genes during rabbit preimplantation development by RT-PCR.

In the future we would like to analyse the expression pattern by Real- Time PCR as well in favor of more efficiency comparison.

The current knowledge on the rabbit pluripotency-associated genes could aid the production of specific antibodies when rabbit cells are examined at the protein level. In addition, the strong conservation of the studied genes indicates that the rabbit would be a valuable model for human preimplantation development. Moreover these isolated genes could play an important role in the analysis, selection of rabbit pluripotent cells and allows generating stable rabbit induced pluripotent stem cell lines.

The identification method of *FGF4, REX1, FBX15, UTF1* pluripotency genes was similar to the description above. The differentiation in this case was that only the partial cDNAs of these genes were identified. Designed primers were used succesfully for the detection of endogenous genes during the rbiPS cell reprogramming.

5.2 Generation of rabbit iPS cells

In the beginning of this experiments there was no available publication about iPS cell generation in rabbit thus we used publications of mouse and rat iPS cells for our work (Takahashi et al. 2006; Li et al. 2009).

Honda et al. were the first group to report the establishment of rbiPSC lines which they produced from adult rabbit liver and stomach cells using lentiviral vectors carrying the human reprogramming genes: *C-MYC, KLF4, SOX2* and *OCT4*. In 2013 Osteil et al also published about generation of rabbit iPS cells using retroviral vector and human reprogramming factors.

In both studies individual gene introduction were used. In our case we created polycistonic vectors in transposon mediated and lentiviral system as well.

Based on our observations we can state that using vectors containing mouse genes the reprogramming of rabbit somatic cells was not efficiency. Hovewer the colonies appeared in 2-3 weeks, but the morphology of the cells was more similar to mouse pluripotent stem cells (round, convex surface, compact colonies) and the cells started to differentiate after some passages.

Using human reprogramming factors the efficiency was already more conspicuous. The apparence of the colonies took a longer time (5- 6 weeks), but the cells in this case were similar to human pluripotent stem cells and was able to be passaged more than 30 passages, altough the generated ips culture seemed to be more and more heterogenous with the increase of passage number.

Based on these observations we could conclude that the „origine" of the reprogramming factors can play a very important role in the reprogramming process.

As the sequence of human reprogramming factors has a higher homology level with rabbit, it was not a surprise that the generation rbiPS cells using human factors was more efficace than the generation with mouse factors in stability.

Based on our result our conclusion was that using species-specific pluripotency genes in reprogramming experiments would be more efficiency. Therefore our newly isolated rabbit pluripotency genes *OCT4, SOX2, KLF4* and *C-MYC* were used for creation of a species-specific reprogramming vector.

By using rabbit specific pluripotency genes we might be also able to reprogram somatic cells and generate iPSCs more efficiently. Future validation of rabbit pluripotent stem cells would benefit greatly from a reliable panel of molecular markers specific to pluripotent cells of the developing rabbit embryo. Our further goal is to produce chimeric rabbit embryos with iPS cells injected into host embryos.

In the future we plan the removal of transgenes from the genarated cell lines because based on our experiencies the presence of the transgenes negativly influence the cell proliferation and differentiation capability.

6. PUBLICATIONS

Publications related to the topic of the dissertation

Book chapter:

- Q. Meng, Zs. Polgar, **Zs. Tancos**, XC. Tian, A. Dinnyes: Cloning of Rabbits. In Principles of Cloning (2nd edition) (Eds: Cibelli, JB, Lanza, R., Campbell, K., West, MD), Academic Press, USA, 2012 Chapter 17. Megjelenése folyamatban.

International journals with impact factors:

- **Zsuzsanna Táncos,** István Bock, Csilla Nemes, Julianna Kobolák and András Dinnyés (2015): Cloning and characterization of rabbit *POU5F1, SOX2, KLF4, C-MYC* and *NANOG* pluripotency-associated genes. *GENE*. doi:10.1016/j.gene.2015.04.034
  IF.: 2.246

- **Zs. Tancos**; Cs. Nemes; Zs. Polgar; E. Gocza; N. Daniel; T.A.E Stout; P. Maraghechi; M. K. Pirity; P. Osteil; Y. Tapponnier; S.Markossian; M. Godet; M. Afanassieff; Zs. Bosze; V. Duranthon; P. Savatier; A. Dinnyes (2012): Generation of rabbit pluripotent stem cell lines. Theriogenology. Published by Elsevier Inc: http://dx.doi.org/10.1016/j.theriogenology.2012.06.017
  IF.: 2.045

- Kiss, K., Kobolak, J., **Tancos, Zs.,** Mamo, S., Polgar, Zs., Rogel-Gaillard, C., Baji Gal, A., and Dinnyes, A. (2008): Promoter analysis of the rabbit pou5f1 gene and its expression in pre-implantation stage embryos. BMC Mol Biol. 2009; 10: 88. Published online 2009 September 4. doi:10.1186/1471-2199-10-88
  IF.: 2.848

National journals with impact factors:

- **Táncos, Zs.** Kobolák, J., Baji Gál, Á., Dinnyés, A. (2010): Pluripotencia gének azonosítása preimplantációs korú nyúlembriókban. Magyar Állatorvosok Lapja, 132. évf. 12. sz. / 2010 707-710 o.
  IF.: 0.300

National journals without impact factors:

- **Táncos Zsuzsanna**, Kobolák Julianna, Baji Gál Árpád, Dinnyés András (2006): Az oct-4 és NANOG transzkripciós faktor gének azonosítása preimplantációs korú nyúl embriókban. Állattenyésztés és Takarmányozás 2006.55.6. 577-589 p.

National abstracts, conferences, posters, presentations:

- **Táncos Zsuzsanna**, Kobolák Julianna, Baji Gál Árpád, Polgár Zsuzsanna, Dinnyés András: Az oct-4 és NANOG transzkripciós faktor gének azonosítása preimplantációs korú nyúl embriókban. **Presentation**:*12. Szaporodásbiológiai Találkozó*, Hajdúszoboszló, 2005. november 4-5.

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