Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Cloning, transgenesis and stem cells

Generation of human primordial germ cell-like from the primed state of pluripotency in Turner syndrome patients

Aline Fernanda de Souza[1,6], Fabiana Fernandes Bressan[1,2], Naira Caroline Godoy Pieri[3], Ramon Botigelli [4], Ester Silveira Ramos[5], Willian Allan King [6], Flávio Vieira Meirelles[1,2]

[1]FZEA, USP - Faculty of Animal Sciences and Food Engineering, University of São Paulo (R. Duque de Caxias, 225 - Jardim Elite, Pirassununga - SP, 13635-900, Brasil); [2]FMVZ,USP - Department of Surgery, Faculty of Veterinary Medicine and Animal Sciences, University of São Paulo (Av. Prof. Orlando Marques de Paiva, 87 - Butantã, São Paulo - SP, 05508-010, Brasil); [3]FMVZ, USP - Swine Research Center, Faculty of Veterinary Medicine and Animal Sciences, University of São Paulo (Av. Prof. Orlando Marques de Paiva, 87 - Butantã, São Paulo - SP, 05508-010, Brasil); [4]UNESP - Institute of Biosciences, Department of Pharmacology, São Paulo State University (Distrito de Rubião Júnior, s/n - Rubião Junior, SP, 18618-970, Brasil); [5]FMRP, USP - Department of Genetic, Medical Faculty of Ribeirão Preto, University of São Paulo (Av. Bandeirantes, 3900 - Vila Monte Alegre, Ribeirão Preto - SP, 14049-900, Brasil); [6]U of G - Department Bioscience, University of Guelph (50 Stone Rd E, Guelph, ON N1G 2W1).

Turner syndrome (TS) is a human genetic disorder in females who lack one X chromosome or some of its parts. The main phenotype of TS is infertility caused by ovarian failure. TS patient-specific iPSCs (TS-iPSCs) may enable disease modelling *in vitro* and investigate genetic and epigenetic factors that influence primordial germ cell (PGCs) development. Herein primordial germ cell-like (hPGCLs) were generated from TS-iPSCs. Peripheral blood mononuclear cells (PBMCs) were collected from five patients and two controls (HCRP-USP, ethics committee no 81683317.1.1001.5440). The inclusion criteria of TS patients were age over 21 years, unrelated, without intellectual disabilities, with TS stigma and karyotype 45, X "pure." Controls met the same criteria however without numerical or structural karyotype aberrations, and hESCs H1 was also used as control. The erythroblast-enriched PBMCs cells were reprogrammed using non-integrative plasmids. All TS and CTRL hiPSCs (passage 10; p10) were characterized by flow cytometry, immunofluorescence, qRT-PCR and *in vitro* differentiation potential (embryoid bodies, EBs). The statistical analyses included an ANOVA followed by multi-comparison Tukey's test ($p<0.001$) using Prism software. Generated hiPSCs lines presented similar self-renewal potential to hESCs H1. No statistical difference among all hiPSCs lines for *OCT4*, *NANOG*, *KLF4*, and *c-MYC* genes was found. All hiPSCs lines formed EBs, however, they differed regarding the *NESTIN* and *NCAM1* genes between TS- and CTRLs hiPSCs lines. Also, TS-hiPSCs Pt.4 was different for the *AFP* gene. There was no difference in *RUNX1*, *CD34*, and *GATA 4* genes. These findings suggest that TS-hiPSC resemble CTRLs hiPSCs and hESCs. Next, we hypothesized that hiPSCs cultured under a feeder-free condition in a primed state of pluripotency could generate hPGCs-like. TS- (p20), CTRLs hiPSCs (p20) and hESCs-H1 (p30) were induced to an epiblast-like cell (EpiLCs) following the protocol by Hayashi et al., (2011, 2012) with minor alterations. EpiLCs were then induced into hPGCs-like following Irie et al., (2015) protocol. hPGCs-like were characterized by cell morphology and by immunofluorescence for OCT4, DPPA3, VASA, DAZL, TF2AC, H3K9me2, H3K27me3, H4K20 and DNMT3B proteins markers. Cell fluorescence was determined by integrated density using Image J software. All TS-hPGCs-like were different from hESCs-H1 hPGCs-like for OCT4, DAZL, and H3K27me3. The TS- hPGCs-like Pt.5 was different from hESCs-PGCs-like for DNMT3B markers. There was no difference for STELLA, VASA, TF2AC, H3K9me2, and H3K20 markers. Herein we show the generation of hPGCs-like independent of X chromosome composition, as TS, enabling further research on genetic/epigenetic mechanisms underlying female infertility. Moreover, these results open the door for future differentiation of TS-PGCs-like into oogonia-like cells.Support: FAPESP 2013/08135-2; 2015/26818-5; 2017/12140-2; 2019/08346-0.

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In vitro pluripotency acquisition in urine-derived cells

Kaiana Recchia[2], Lucas S. Machado[2], Naíra C. G. Pieri[3], Ramon C. Botigelli[4], Gabriela Barbosa[3], Laís V. F. Pêssoa[3], Paulo F. Neto[3], Simone M. M. K. Martins[3], Raquel V. G. Castro[5], Flávio V. Meirelles[3,2], Fabiana F. Bressan[2,3]

[2]Faculdade de Medicina Veterinária e Zootecnia - FMVZ - USP (Av. Prof. Orlando Marques de Paiva, 87 - Butantã, São Paulo - SP); [3]Faculdade de Zootecnia e Engenharia de Alimentos - FZEA - USP (R. Duque de Caxias, 225 - Jardim Elite, Pirassununga - SP); [4]Instituto de Biociência - IBB - UNESP (Dr. Antônio Celso Wagner Zanin, 250 - Distrito de Rubião Junior - Botucatu/SP); [5]Faculdade de Ciências Agrárias e Veterinárias - FCAV - UNESP (Via de Acesso Professor Paulo Donato Castelane Castellane S/N - Vila Industrial, Jaboticabal - SP).

Innovation and improvement in animal production or regenerative medicine are widely desired, and the swine is an adequate model in this context. The non-invasive collection of cells and its reprogramming into a pluripotent state is advantageous and would enable the easily accessible generation of *in vitro* stem cells. Herein we aimed to isolate urine-derived cells (UDCs), *in vitro* culture and reprogram them into iPSCs (induced pluripotent stem cells). For that, the urine was collected through spontaneous urination from three females, the precipitated was washed and cultured in 45% DMEM high glucose (Gibco), 5% FBS, 50% REBM media, REGM supplements: hEGF, insulin, hydrocortisone, GA-1000, FBS, transferrin, triiodothyronine, epinephrine (Lonza) and supplemented with 1% glutamine, 1% MEM neaa, 1% pen/strep (all Gibco) 2.5ng/mL bFGF (Peprotech). After one week in culture, the UDCs were observed in colonies with epithelial-like morphology. One UDC lineage was tested by flow cytometry for mesenchymal marker CD44 (87.3% positive) and vimentin epithelial marker (negative). At passage 2 the UDCs were transduced with a lentiviral vector containing murine transcription factors (mOSKM – OCT4, SOX2, KLF4, and c-MYC), and after 5 days the cells were plated onto MEFs and cultured with KnockOut DMEM/F12 medium, KnockOut Serum Replacement, MEM NEAA, L-Glutamine, 2-Mercaptoethanol and pen/strep (all Gibco) supplemented with 10ng/ml bFGF (Peprotech). Approximately 12 days after transduction colonies were observed, and reprogramming efficiency was 8.45% (number of iPSCs colonies per number of cells plated for transduction). Eight clonal lines were isolated and further cultured, and 3 lineages were further maintained in culture (C1, C2, and C3) for at least 32, 28, and 31 passages, respectively. All three lines were positive for alkaline phosphatase in early and intermediate passages and formed embryonic bodies. The lines were also tested for pluripotent genes NANOG, OCT4, SOX2, SSEA1, and TRA1 81 by immunocytochemistry. C1, C2, and C3 were positive only for OCT4, SOX2, NANOG, and SSEA1; however, the line C3 was partially positive for TRA1 81. The expression of endogenous pluripotent genes was evaluated using RT-qPCR for the OCT4, SOX2, and NANOG genes, and analyzed in early and late passage groups for each line. Statistical analysis revealed differences between passages for NANOG expression, and between colonies for SOX2 expression. However, throughout the culture, the cells maintained mOSKM expression demonstrating the non-silencing of the exogenous vector. In conclusion, this study presents the generation of partially reprogrammed iPSCs from urine-derived cells in the swine model, an unprecedented result in the scientific literature enabling its use in studies for animal production and regenerative medicine. Financial Support: FAPESP (2019/02811- 2, 2015/26818-5), CNPq 433133/2018-0, and CAPES.

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Cloning, transgenesis and stem cells

Concomitant usage of chromatin reprogramming molecules increase bovine fibroblasts plasticity through heterochromatin remodeling

Dewison Ricardo Ambrizi, Rafael Vilar Sampaio, Juliano Rodrigues Sangalli, Jessica Brunhara Cruz, Laís Vicari de Figueiredo Pessôa, Ricardo Perecin Nociti, abiana Fernandes Bressan, Flavio Vieira Meirelles

USP - Universidade de São Paulo (Pirassununga - SP, Rua Duque de caxias Norte 225 - Jardim Elite, CEP: 13635-900)

The nuclear organization of higher eukaryotes is composed of 3D complex structure maintained by DNA and associated proteins constituting the chromatin. However, during the cellular reprogramming process (iPSC and nuclear transfer), these marks confer resistance to successful reprogramming. Small molecules compounds can inhibit enzymes responsible for these epigenetic marks, alleviate the chromatin compaction, and facilitate the cellular reprogramming process in mice. Nevertheless, the use of single molecules has not proven to be useful in bovines so far, suggesting that a combination of molecules may be necessary to circumvent these epigenetic barriers. Here, we aimed to combine several small molecules compounds to create a cocktail targeting all these marks at the same time and investigate the effect of cell culture under this medium. We cultured bovine fibroblasts using stem cell culture medium (DMEM F12 Knockout with 15% KSR) for the control group. Treated group was supplemented with trichostatin (10nM), sodium butyrate (0.25mM), chaetocin (5nM), UNC0638 (250nM), and GSK343 (100nM). We cultured cells from control and treated groups for 4 (D4) and 8 (D8) days. In D4 we observed some cells aggregating and forming small rounded-shape structures resembling stem cell colonies. At the D8, these structures were bigger and spherical (n=3), suggesting the cells were transdifferentiating or chemically reprogramming. To gain insights about the molecular signature of these chemically treated cells, we extracted RNA and evaluated the expression of genes related to pluripotency or deposition of epigenetic marks in these cells. In order to evaluate pluripotent genes total RNA was extracted using Trizol (Invitrogen). The amount was normalized and briefly converted to cDNA using the High-Capacity Kit (Life Tech). With the same input, we carried out RT-PCR for the target genes and normalized the expression using the geometrical mean of 3 reference genes (RPL15, ACTB and H2A) using the ΔΔCT method. As a result, the pluripotency genes Nestin and POU5F1 exhibited a significant increase in the treated group comparing with control group in D4 (2.2 and 4.7) and D8 (2.8 and 6 fold change). The Nanog, a key marker of pluripotency, increased in D8 (4 fold change), suggesting a prolonged time of culture may be necessary to reactivate some genes. Chromatin modifying enzymes such as SUV39H1, G9a, HDAC1, HDAC2 and DNMT1 also increased gene expression in D8 treated-cells. Altogether, these results demonstrate that the culture of fibroblast cells in a cocktail can increase pluripotency gene expression, modulate the mRNA levels epigenetic enzymes, and cause alterations on cell morphology. In the future, approaches similar to the used herein can propitiate the generation of stem cells chemically induced or improved other correlated biotechnologies such as mammalian cloning, modulating the cellular epigenetic marks.Financial Support: CEPID 2013/08135-2; FAPESP 2018/15089-0