Derivation of ringed seal (*Phoca hispida*) tripotent induced pluripotent stem-like cells

Violetta R. Beklemisheva[1], Aleksei G. Menzorov[2,3*]

* Corresponding author: Aleksei G. Menzorov, menzorov@bionet.nsc.ru

[1]Laboratory of Animal Cytogenetics, Institute of Molecular and Cellular Biology of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia

[2]Sector of Cell Collections, Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia

[3]Natural Sciences Department, Novosibirsk State University, Novosibirsk, Russia

Abstract

Induced pluripotent stem (iPS) cells have been produced just for a few species among order Carnivora: snow leopard, Bengal tiger, serval, jaguar, cat, dog, ferret, and American mink. For the first time, we derived the ringed seal (*Phoca hispida*) iPS-like cells. We had shown the expression of pluripotency marker gene *Rex1*. Ringed seal iPS-like cells were able to differentiate into derivatives of endoderm (expression of *AFP*), mesoderm (adipocytes and osteocytes), and trophectoderm (expression of *Cdx2*). To confirm pluripotency, we need to differentiate cells into ectoderm cell types, for instance into neurons.

Keywords

pluripotency, reprogramming, ringed seal, iPS cells

INTRODUCTION

Order Carnivora consists of two suborders: Caniformia and Feliformia. It includes domestic animals, that are used for disease modeling, fur-bearing animals, such as mink, marine mammals, and other species. Derivation of induced pluripotent stem (iPS) cells could allow insights into pluripotency and embryonic development of these species, as well as the development of new disease models. Currently, pluripotent cells were produced from several Carnivora species: dog (Luo et al., 2011; Baird et al., 2015; Lee et al., 2011; Vaags et al., 2009; Shimada et al., 2010; Whitworth et al., 2012; Koh et al., 2012; Tsukamoto et al., 2018), snow leopard (Verma et al., 2012), Bengal tiger, serval, jaguar, and cat (Verma et al., 2013; Dutton et al., 2019; Gómez et al., 2010), ferret (Gao et al., 2020), and American mink (Menzorov et al., 2015).

Marine mammals represent the basal Carnivora group. Their iPS cells would expand our knowledge of embryonic development and differentiation. We report the generation of ringed seal (*P. hispida*) iPS-like cells. These cells expressed pluripotency marker *Rex1* and were able to differentiate into endo-, meso-, and trophectoderm.

MATERIALS AND METHODS

Production of ringed seal fibroblasts

Primary fibroblasts of the ringed seal were obtained from lung necropsy. Tissue samples from wild female were collected during aboriginal quota sealing in the coastal waters of the Bering Sea (Mechigmen bay, Chukotka Autonomous Okrug, Russia). To establish primary

fibroblast's cell culture, we used conventional technique (Stanyon, Galleni, 1991). The fibroblast culture medium consisted of a-MEM supplemented with 15 % fetal bovine serum (FBS), 1x MEM non-essential amino acids solution, 1x GlutaMAX supplement, and 1x penicillin-streptomycin (Thermo Fisher Scientific, USA).

Production of ringed seal iPS cell lines

To produce iPS cells from the ringed seal fibroblasts we used lentiviral vectors LeGO (http://www.lentigo-vectors.de/vectors.htm) with EGFP and human reprogramming transcription factors: OCT4, SOX2, C-MYC and KLF4, courtesy of Dr. Sergei L. Kiselev, Moscow. We used previously the published protocol (Menzorov et al., 2015) with minor modifications. Lentiviruses were produced in the Phoenix cell line using Lipofectamine 3000 (Thermo Fisher Scientific, USA). The multiplicity of infection was estimated as 5.1 using EGFP lentiviral vector (Beklemisheva, Menzorov, 2018). Fibroblasts (3 x 10^5 cells, 30 x 10^3 cells/cm^2) plated the day before were transduced with viruses containing four reprogramming transcription factors: 50 % virus supernatant, 50 % fibroblast culture medium without antibiotics with heat-inactivated FBS, and 10 µg/ml Polybrene. Transduction was performed two consecutive days. Cells were passaged onto a 6 cm cell culture dish coated with 0.1 % gelatin on mouse strain CD-1 feeder cells on day 5. From day 6 we used iPS cell culture medium: a-MEM supplemented with 20 % ES cell qualified FBS, 1x MEM non-essential amino acids solution, 1x GlutaMAX supplement, 0.1 mM 2-mercaptoethanol, 1x penicillin-streptomycin, and 10 ng/ml bFGF recombinant human protein (Thermo Fisher Scientific, USA). From day 6 until day 12 the medium was changed once in two days with the addition of 1 mM valproic acid (Sigma-Aldrich, USA). On day 23 colonies were picked up and expanded on the feeder. The passage was performed with 0.25 % Trypsin-EDTA (Thermo Fisher Scientific, USA). All cell cultures were maintained at 37°C and 5 % CO_2.

iPS cell derivation was performed at the Collective Center of ICG SB RAS "Collection of Pluripotent Human and Mammalian Cell Cultures for Biological and Biomedical Research" (http://ckp.icgen.ru/cells/; http://www.biores.cytogen.ru/icg_sb_ras_cell/).

All animal studies were undertaken with prior approval from the Interinstitutional Bioethical Committee of the Institute of Cytology and Genetics SB RAS and the Ethics Committee on Animal and Human Research of the Institute of Molecular and Cellular Biology SB RAS, Russia (order No. 32 of 5 May 2017).

Cytogenetic analysis

Cytogenetic analysis for fibroblasts was carried out on passage 7 and for iPS-like cell line on passage 9. The preparation of metaphase chromosomes from fibroblasts was performed as previously described (Yang et al., 1999; Graphodatsky et al., 2000; Graphodatsky et al., 2001). GTG-banding of metaphase chromosomes was done according to a previously published protocol (Seabright, 1971). For each cell line, an average of 50 metaphase plates were analyzed using the VideoTest system (Saint Petersburg, Russia) with a CCD camera (Jenoptik, Germany) mounted on a Zeiss microscope Axioscope 2 (Zeiss, Germany). Metaphase spreads images were edited in Corel Paint Shop Pro Photo X2 (Corel, Canada). Chromosomes of the ringed seal (*P. hispida*) were arranged according to the nomenclature (Graphodatsky et al., 2020).

iPS cell differentiation

Differentiation into embryoid bodies (EBs) was performed in EB differentiation medium, the same composition as iPS cell culture medium but with 20 % FBS instead of ES qualified FBS and without bFGF. Cells were passaged into 1 % agarose coated cell culture plates. The medium was changed every second day for 31 days. On day 5 part of EBs were plated onto 0.1 % gelatin-coated cell culture plates for osteocyte and adipocyte differentiation. Osteocyte differentiation was carried out in EB differentiation medium; adipocyte differentiation in a similar adipocyte differentiation medium supplemented with 10 % knockout serum replacement (KSR) (Thermo Fisher Scientific, USA) instead of FBS from day 6.

Cytochemical staining

Cells were fixed by 4 % paraformaldehyde for 20 min and washed with PBS. Adipocytes were stained with 0.7 % Sudan black B in propylene glycol, washed twice with 0.85 % propylene glycol, and washed multiple times with PBS. Calcification was shown by staining with alizarin red (Sigma-Aldrich) according to the manufacturer's recommendations. Staining was analyzed on Zeiss Observer.Z1 fluorescent microscope with AxioCam HRm 3 CCD-camera (Zeiss, Germany). Digital images were analyzed using the ZEN 2 starter (Zeiss, Germany) software.

DNA isolation

Genomic DNA was isolated from cells using a PCR buffer with nonionic detergents (PBND), which was adapted from a protocol from Perkin Elmer Cetus (Higuchi, 1989).

RNA isolation and cDNA synthesis

RNA was isolated using Aurum Total RNA mini kit (Bio-Rad, USA). Genomic DNA was removed using *DNase*I (Fermentas, USA), 0.4 micrograms of total RNA was used for cDNA synthesis by First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA).

Primer design and PCR

We used Primer-BLAST software (Ye et al., 2012) to design primers for mink *Rex1* and canine *Cdx2*: qNvRex1F 5'-AAA GCG TTT TCC ACA CCC CT-3', qNvRex1R 5'-CTC CTT GTC CAT GGT CCT CG-3', CfCdx2E1F 5'-GGA ACC TGT GCG AGT GGA TG-3', and CfCdx2E3R 5'-TTC CTT TCC TTG GCT CTG CG-3'.

We used previously published primer sequences for human-specific *KLF4* transgene (Mathew et al., 2010), mycoplasma detection (Choppa et al., 1998), mink *Hprt1* (Rouvinen-Watt et al., 2012), mink *Oct4* (Menzorov et al., 2015), and human *AFP* (Huangfu et al., 2008).

PCR was performed using BioMaster HS-Taq PCR-Color (2×) (Biolabmix, Russia) in 10 µL reaction volume.

RESULTS

iPS cell derivation and differentiation

Experiment outline is shown in Figure 1, A. To produce iPS cells from primary ringed seal fibroblasts we used the following human reprogramming transcription factors: OCT4, SOX2, C-MYC, and KLF4. On day 26 after transduction, there were colonies with different morphology. Two colonies were picked up and expanded. One of them, iPHIS1, gave rise to cells with epithelial-like morphology (Fig. 1, B) that grew in a monolayer. Overgrown culture formed "bubbles" (Fig. 1, C) that later detached and floated as cyst-like structures resembling canine embryos (Hayes et al., 2008).

We attempted to differentiate iPHIS1 cells into derivatives of the three germ layers. Solid and cyst-like EBs were formed after passage to non-adhesive culture plates (Fig. 1, E). EB gene expression analysis is described below. Adipo- and osteogenic differentiation was successfully performed in the adipo- and EB differentiation media, respectively (Fig. 1, H, I). Interestingly, there was some calcification in the adipocyte differentiation medium (data not shown).

Cytogenetic analysis

We performed the cytogenetic analysis of the ringed seal primary fibroblasts and iPHIS1 cells. Fibroblasts had 32 chromosomes, XX (n = 50) (Fig. 1, D) with 15.7 % polyploid cells. iPHIS1

cells (n = 50) had 32 (n = 47), 33 (n = 2), and 35 (n = 1) chromosomes, with 5 % polyploid cells. We had not revealed a difference in the pattern of GTG-banding between karyotypes of the primary fibroblasts and iPHIS1 cells. We conclude that the iPHIS1 karyotype is stable.

iPS cell and EB gene expression

We were able to show expression of one of the key pluripotency markers *Rex1* (*Zfp42*) in iPHIS1 cells (Fig. 1, F), as well as *Oct4*. There also was a weak expression of *Nanog* (data not shown). We were not able to determine its expression level as qualitative PCR was performed, though we previously showed a very low level of *Nanog* expression in American mink pluripotent stem cells (Menzorov et al., 2015). Importantly, we were not able to amplify human *KLF4* transgene (Fig. 1, F), it suggests transgene silencing.

Analysis of EBs revealed the expression of *AFP*, endoderm marker, and *Cdx2*, trophoblast marker (Fig. 1, G). Ectoderm marker, *Tubb3*, was expressed in both iPHIS1 cells and embryoid bodies (data not shown).

Ringed seal fibroblasts and iPHIS1 cells were negative for *Mycoplasma* contamination.

DISCUSSION

Transcription factors Oct4, Sox2, c-Myc, and Klf4 were used to generate iPS cells from a variety of Carnivora species. We decided to produce iPS cells from the ringed seal (*P. hispida*), the basal representative of the suborder Caniformia. Only one cell line expressed pluripotency marker genes *Rex1* and *Oct4*. Its morphology differed from mouse, human, and mink iPS cells (Fig. 1, B). Similar to American mink iPS cells, overgrown cell culture formed “bubbles” (Fig. 1, C) that later unfastened and floated as cysts. We differentiated iPHIS1 cells into EBs to analyze differentiation potential (Fig. 1, E). Cyst-like EBs resembled canine embryos (Hayes et al., 2008), expressed endoderm marker *AFP* and trophoblast marker *Cdx2* (Fig. 1, G). EBs seated on gelatin differentiated into mesoderm derivatives: adipo- and osteocytes. We were not able to show ectoderm differentiation, as *Tubb3* was expressed in both iPHIS1 and EBs. The presence of *Tubb3* transcript in undifferentiated pluripotent stem cells is in accordance with our previous data on mouse ES cells (Menzorov et al., 2019). Quantitative PCR or another ectoderm marker and/or different differentiation conditions should solve this problem.

Another potential sign of the iPHIS1 pluripotency is human *KLF4* transgene silencing (Fig. 1, F). Viral transgene silencing in pluripotent stem cells is a well-known phenomenon (Maherali et al., 2007; Wernig et al., 2007; Okita et al., 2007).

One of the interesting properties of iPHIS1 cell line is its ability to differentiate into trophoblast, as shown by *Cdx2* expression. Trophoblast differentiation was shown for human and mouse primed pluripotent stem cells (Xu et al., 2002; Kojima et al., 2014) and canine iPS cells (Luo et al., 2011; Wilcox et al., 2008). Differentiation into trophoblast suggests a primed pluripotency state of iPHIS1.

We used bFGF to derive and propagate the iPHIS1 cell line. Colonies with iPS-like morphology were not formed in the media supplemented with LIF or combination of LIF and bFGF. Canine pluripotent stem cells were produced with LIF (Luo et al., 2011; Baird et al., 2015; Lee et al., 2011; Vaags et al., 2009) or LIF and bFGF (Shimada et al., 2010; Whitworth et al., 2012; Koh et al., 2012; Tsukamoto et al., 2018). Also, cells were later cultured in the presence of LIF only (Whitworth et al., 2012). Other Carnivora species pluripotent cells include snow leopard (Verma et al., 2012), Bengal tiger, serval, jaguar, and cat (Verma et al., 2013; Dutton et al., 2019) cultured with LIF; cat cultured with LIF and bFGF (Gómez et al., 2010), and ferret cultured with bFGF (Gao et al., 2020). Interestingly, cat iPS cells required species-specific feline bFGF (Dutton et al., 2019). We generated American mink iPS cells without LIF of bFGF supplementation (Menzorov et al., 2015), though inactivated mouse embryonic fibroblasts secret both growth factors. Different requirements of the iPS cell culture of various species indicate that different signaling pathways are activated. It leads to different pluripotency states, naïve, primed, or other, as additional distinct pluripotency states had been described recently. More high-quality transcriptome data may facilitate the distinction between various pluripotency states in different species.

Pluripotent stem cells of different pluripotency status also have distinctive morphology. American mink pluripotent stem cells form colonies unlike mouse and human naïve and primed cells (Menzorov et al., 2015). Ringed seal iPHIS1 cell line is at least multipotent and also have different morphology.

We produced only one ringed seal iPS-like cell line, other experiments were not successful. Suboptimal culture conditions might be the main reason. ES cell qualified FBS batch was only tested for mouse ES cell growth support. Also, growth factor origin may be important. We used human bFGF, and for cat iPS cells only species-specific feline LIF was able to support pluripotency (Dutton et al., 2019). Also, growth factor concentration is an important factor. Ferret iPS cells were derived with human bFGF, but in 10x concentration compared to human iPS cells and this study (Gao et al., 2020). Different researchers used small molecules to enhance iPS cell derivation efficiency. In our experience, 2i inhibitors (PD0325901 and

CHIR99021) and TGH-β antagonist A83-01 caused substantial fibroblast death, thus we were not able to use them. Another way to increase efficiency is to use different transgenes and/or delivery vectors. We have recently shown that Sendai virus-based vector transgene expression level is superior to lentiviruses (Beklemisheva, Menzorov, 2018). Also, another set of reprogramming factors was successfully used to reprogram not only human, but ferret fibroblasts to pluripotency, OCT4, SOX2, KLF4, L-MYC, LIN28A, mp53DD, and 160 oriP/EBNA-1 (Gao et al., 2020).

CONCLUSIONS

We produced tri-potent ringed seal iPS-like cells, capable of differentiation into endoderm, mesoderm, and trophoblast. We need to achieve ectoderm differentiation to prove pluripotency.

ACKNOWLEDGMENT

The reported study was funded by RFBR, project number 20-04-00369, and the Ministry of Education and Science of Russia, state project 0324-2019-0041-C-01.

Authors are thankful to Michael Zelensky, Alexey Ottoj, and the Community of the Chukotka Autonomous Region indigenous “Lorino” (Russian Federation) for assistance in the ringed seal tissue sample collection. The authors gratefully acknowledge the primary fibroblast cell culture of *P. hispida* provided by the “Molecular and Cellular Biology” core facility of the Institute of Molecular and Cellular Biology SB RAS.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

VRB obtained ringed seal fibroblasts and performed cytogenetic analysis. AGM produced and differentiated iPS cells, performed gene expression analysis. AGM carried out interpretations of the data and project coordination; AGM did most of the writing with contributions from VRB. All authors read and approved the final manuscript.

REFERENCES

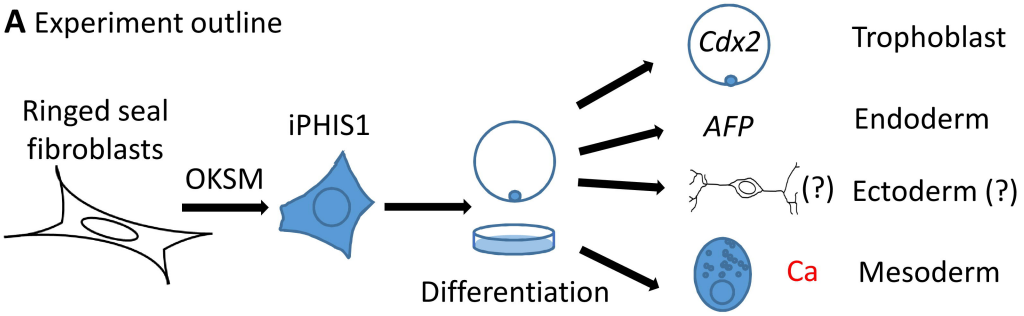
1. Baird A, Barsby T, Guest DJ. Derivation of Canine Induced Pluripotent Stem Cells. Reprod Domest Anim. 2015 Aug;50(4):669-76. doi: 10.1111/rda.12562
2. Beklemisheva V.R., Menzorov A.G. Use of a Sendai virus-based vector for efficient transduction of pinniped fibroblasts. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2018;22(8):1020-1025. DOI 10.18699/VJ18.445 (in Russian)
3. Choppa PC, Vojdani A, Tagle C, Andrin R, Magtoto L. Multiplex PCR for the detection of Mycoplasma fermentans, M. hominis and M. penetrans in cell cultures and blood samples of patients with chronic fatigue syndrome. Mol Cell Probes. 1998 Oct;12(5):301-8.
4. Dutton L, Dudhia J, Guest DJ, Connolly DJ. Inducing pluripotency in the domestic cat (Felis Catus). Stem Cells Dev. 2019 Aug 7. doi: 10.1089/scd.2019.0142
5. Gao J, Petraki S, Sun X, Brooks LA, Lynch TJ, Hsieh C-L, Elteriefi R, Lorenzana Z, Punj V, Engelhardt JF, Parekh KR, Ryan AL. Derivation of Induced Pluripotent Stem Cells From Ferret Somatic Cells. Am J Physiol Lung Cell Mol Physiol. 2020 Apr 1;318(4):L671-L683. doi: 10.1152/ajplung.00456.2019
6. Gómez MC, Serrano MA, Pope CE, Jenkins JA, Biancardi MN, López M, Dumas C, Galiguis J, Dresser BL. Derivation of cat embryonic stem-like cells from in vitro-produced blastocysts on homologous and heterologous feeder cells. Theriogenology. 2010 Sep 1;74(4):498-515. doi: 10.1016/j.theriogenology.2010.05.023
7. Graphodatsky AS, Sablina OV, Meyer MN, Malikov VG, Isakova EA, Trifonov VA, Polyakov AV, Lushnikova TP, Vorobieva NV, Serdyukova NA, Perelman PL, Borodin PM, Benda P, Frynta D, Leikepová L, Munclinger P, Piálek J, Sádlová J, Zima J. Comparative Cytogenetics of Hamsters of the Genus Calomyscus. Cytogenet Cell Genet. 2000;88(3-4):296-304. doi: 10.1159/000015513
8. Graphodatsky AS, Yang F, O'Brien PC, Perelman P, Milne BS, Serdukova N, Kawada SI, Ferguson-Smith MA. Phylogenetic Implications of the 38 Putative Ancestral Chromosome Segments for Four Canid Species. Cytogenet Cell Genet. 2001;92(3-4):243-7. doi: 10.1159/000056911
9. Hayes B, Fagerlie SR, Ramakrishnan A, Baran S, Harkey M, Graf L, Bar M, Bendoraite A, Tewari M, Torok-Storb B. Derivation, characterization, and in vitro differentiation of canine embryonic stem cells. Stem Cells. 2008 Feb;26(2):465-73.

10. Higuchi R. Rapid, efficient DNA extraction for PCR from cells or blood. Amplifications (Perkin Elmer Cetus) 1989;2:1–3.
11. Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, Chen S, Muhlestein W, Melton DA. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol. 2008 Nov;26(11):1269-75. doi: 10.1038/nbt.1502
12. Koh S, Thomas R, Tsai S, Bischoff S, Lim J-H, Breen M, et al: Growth requirements and chromosomal instability of induced pluripotent stem cells (iPSC) generated from adult canine fibroblasts. Stem Cells Dev. 2012, 22 (6): 951-963.
13. Kojima Y, Kaufman-Francis K, Studdert JB, Steiner KA, Power MD, Loebel DA, Jones V, Hor A, de Alencastro G, Logan GJ, Teber ET, Tam OH, Stutz MD, Alexander IE, Pickett HA, Tam PP. The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. Cell Stem Cell. 2014 Jan 2;14(1):107-20. doi: 10.1016/j.stem.2013.09.014
14. Lee AS1, Xu D, Plews JR, Nguyen PK, Nag D, Lyons JK, Han L, Hu S, Lan F, Liu J, Huang M, Narsinh KH, Long CT, de Almeida PE, Levi B, Kooreman N, Bangs C, Pacharinsak C, Ikeno F, Yeung AC, Gambhir SS, Robbins RC, Longaker MT, Wu JC. Preclinical derivation and imaging of autologously transplanted canine induced pluripotent stem cells. J Biol Chem. 2011 Sep 16;286(37):32697-704. doi: 10.1074/jbc.M111.235739
15. Luo J, Suhr ST, Chang EA, Wang K, Ross PJ, Nelson LL, Venta PJ, Knott JG, Cibelli JB. Generation of leukemia inhibitory factor and basic fibroblast growth factor-dependent induced pluripotent stem cells from canine adult somatic cells. Stem Cells Dev. 2011 Oct;20(10):1669-78. doi: 10.1089/scd.2011.0127.
16. Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, Plath K, Hochedlinger K. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell. 2007 Jun 7;1(1):55-70. doi: 10.1016/j.stem.2007.05.014
17. Mathew R, Jia W, Sharma A, Zhao Y, Clarke LE, Cheng X, Wang H, Salli U, Vrana KE, Robertson GP, Zhu J, Wang S. Robust activation of the human but not mouse telomerase gene during the induction of pluripotency. FASEB J. 2010 Aug;24(8):2702-15. doi: 10.1096/fj.09-148973
18. Menzorov AG, Matveeva NM, Markakis MN, Fishman VS, Christensen K, Khabarova AA, Pristyazhnyuk IE, Kizilova EA, Cirera S, Anistoroaei R, Serov OL. Comparison of American

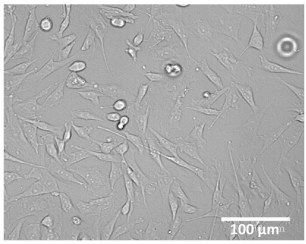
mink embryonic stem and induced pluripotent stem cell transcriptomes. BMC Genomics. 2015;16 Suppl 13:S6. doi: 10.1186/1471-2164-16-S13-S6

19. Menzorov AG, Orishchenko KE, Fishman VS, Shevtsova AA, Mungalov RV, Pristyazhnyuk IE, Kizilova EA, Matveeva NM, Alenina N, Bader M, Rubtsov NB, Serov OL. Targeted genomic integration of EGFP under tubulin beta 3 class III promoter and mEos2 under tryptophan hydroxylase 2 promoter does not produce sufficient levels of reporter gene expression. J Cell Biochem. 2019 Oct;120(10):17208-17218. doi: 10.1002/jcb.28981
20. Graphodatsky AS, Perelman PL, O'Brien SJ. Atlas of Mammalian Chromosomes, 2nd Edition. Wiley-Blackwell, 2020:699.
21. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature. 2007 Jul 19;448(7151):313-7.
22. Rouvinen-Watt K, Harris L, Dick M, Pal C, Lei S, Mustonen AM, Nieminen P. Role of hepatic de novo lipogenesis in the development of fasting-induced fatty liver in the American mink (Neovison vison). Br J Nutr. 2012 Oct 28;108(8):1360-70. doi: 10.1017/S0007114511006775
23. Seabright M. A Rapid Banding Technique for Human Chromosomes. Lancet. 1971 Oct 30;2(7731):971-2. doi: 10.1016/s0140-6736(71)90287-x
24. Shimada H, Nakada A, Hashimoto Y, Shigeno K, Shionoya Y, Nakamura T. Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. Mol Reprod Dev. 2010 Jan;77(1):2. doi: 10.1002/mrd.21117
25. Stanyon R, Galleni L. A rapid fibroblast culturetechnique for high resolution karyotypes. Italian Journal of Zoology. 1991,58(1):81-83. doi:10.1080/11250009109355732
26. Tsukamoto M, Nishimura T, Yodoe K, Kanegi R, Tsujimoto Y, Alam ME, Kuramochi M, Kuwamura M, Ohtaka M, Nishimura K, Nakanishi M, Inaba T, Sugiura K, Hatoya S. Generation of Footprint-Free Canine Induced Pluripotent Stem Cells Using Auto-Erasable Sendai Virus Vector. Stem Cells Dev. 2018 Nov 15;27(22):1577-1586. doi: 10.1089/scd.2018.0084
27. Vaags AK, Rosic-Kablar S, Gartley CJ, Zheng YZ, Chesney A, Villagómez DA, Kruth SA, Hough MR. Derivation and characterization of canine embryonic stem cell lines with in vitro and in vivo differentiation potential. Stem Cells. 2009 Feb;27(2):329-40. doi: 10.1634/stemcells.2008-0433

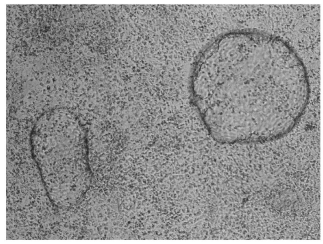
28. Verma R, Holland MK, Temple-Smith P, Verma PJ. Inducing pluripotency in somatic cells from the snow leopard (Panthera uncia), an endangered felid. Theriogenology. 2012 Jan 1;77(1):220-8, 228.e1-2. doi: 10.1016/j.theriogenology.2011.09.022
29. Verma R, Liu J, Holland MK, Temple-Smith P, Williamson M, Verma PJ. Nanog is an essential factor for induction of pluripotency in somatic cells from endangered felids. Biores Open Access. 2013 Feb;2(1):72-6. doi: 10.1089/biores.2012.0297
30. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature. 2007 Jul 19;448(7151):318-24.
31. Whitworth DJ, Ovchinnikov DA, Wolvetang EJ: Generation and Characterization of LIF-dependent Canine Induced Pluripotent Stem Cells from Adult Dermal Fibroblasts. Stem Cells Dev. 2012, 21 (12): 2288-2297.
32. Wilcox JT, Semple E, Gartley C, Brisson BA, Perrault SD, Villagómez DA, Tayade C, Becker S, Lanza R, Betts DH. Characterization of canine embryonic stem cell lines derived from different niche microenvironments. Stem Cells Dev. 2009 Oct;18(8):1167-78. doi: 10.1089/scd.2008.0336
33. Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA. BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol. 2002 Dec;20(12):1261-4.
34. Yang F, O'Brien PC, Milne BS, Graphodatsky AS, Solanky N, Trifonov V, Rens W, Sargan D, Ferguson-Smith MA. A Complete Comparative Chromosome Map for the Dog, Red Fox, and Human and Its Integration With Canine Genetic Maps. Genomics 1999 Dec 1;62(2):189-202. doi: 10.1006/geno.1999.5989
35. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 2012 Jun 18;13:134. doi: 10.1186/1471-2105-13-134.



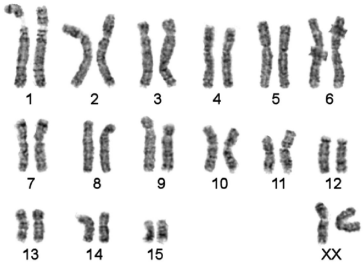
B iPHIS1 on gelatin without feeder



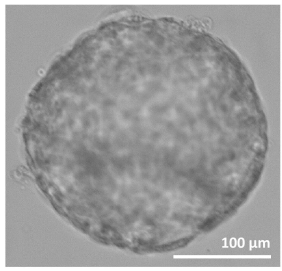
C Overgrown iPHIS1 forms "bubbles", similar to mink iPS cells



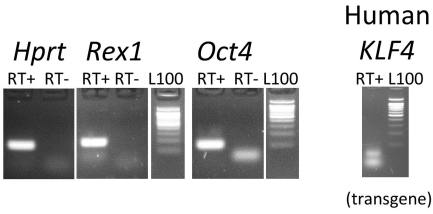
D iPHIS1 karyotype



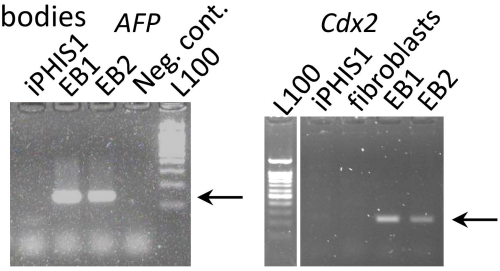
E iPHIS1 embryoid body on day 17



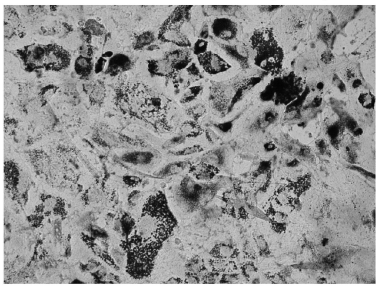
F iPHIS1 gene expression



G Gene expression in embryoid bodies



H Differentiation into adipocytes



I Differentiation into osteocytes

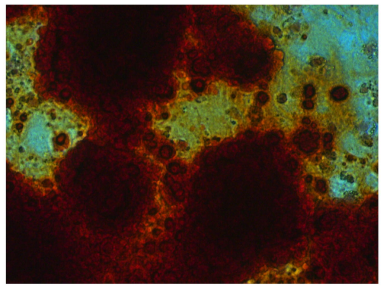


Figure 1. Derivation and characterization of ringed seal tripotent induced pluripotent stem-like cells