RABBIT PLURIPOTENT STEM CELLS: A PROMISING AND USEFUL BIOTEHNOLOGY TOOL

Afanassieff M.[1]*, Gavin-Plagne L.[1,2], Perold F.[1], Bouchereau W.[1], Buff S.[2], Joly T.[2], Rival-Gervier S.[1], Beaujean N.[1]

[1]Univ Lyon, Université Claude Bernard Lyon 1, Inserm, INRAE, Stem cell and Brain Research Institute U1208, USC1361, 18 Avenue du Doyen Lépine F-69500 Bron, France
[2]Univ Lyon, Université Claude Bernard Lyon 1, VetAgro Sup, ISARA-Lyon, UPSP ICE 2016.A104, 1 Avenue de Bourgelat, F-69280 Marcy l'Etoile, France
*Corresponding author: marielle.afanassieff@inserm.fr

ABSTRACT

Embryo-derived (ESCs) and induced pluripotent stem cells (iPSCs) are both useful biotechnology tools in mice and promising therapeutic agents in humans. In rodents, they made it possible to develop animal models thanks to their ability to produce somatic and germline chimaera. In Humans, they are already involved in clinical trials for several degenerative diseases. Rabbit pluripotent stem cells (rbPSCs) differ from their rodent counterparts but are close to their primate counterparts. In the first part of this article, we describe the state of art of these rabbit cells as well as their future use in biotechnologies for the creation of organoids, bioreactors or animal models of human diseases and for the conservation of biodiversity. In the second part, we present the cryopreservation protocol we have developed in order to freeze rbPSCs without any animal products for health safety reasons.

Key words: Rabbit, Pluripotency, ESC, iPSC, Animal models, Organoids, Biodiversity, Cryopreservation.

INTRODUCTION

Pluripotency defines the ability of a stem cell to differentiate into three germ layers: ectoderm, mesoderm, and endoderm. In mice, pluripotent stem cells (PSCs) exist in three forms: the embryonic stem cells (ESCs) and the epiblast stem cells (EpiSCs), both derived from embryos; and the induced pluripotent stem cells (iPSCs) produced by somatic cell reprogramming. Both ESCs and iPSCs exploit LIF/STAT3 signaling to maintain robust self-renewal. In contrast, self-renewal of EpiSCs depends on FGF2 and Activin signaling pathways, leading to genetic instability and a high propensity to differentiation. Moreover, only the ESCs and iPSCs can colonize the blastocyst following embryo microinjection and contribute to the development of all tissue types including the germ cells. In contrast, EpiSCs fail to colonize the blastocyst, consequently, are not suitable for generation of transgenic mice. It is believed that these differences are largely due to differences in their developmental maturity: ESCs and iPSCs represent a pristine state termed as the naïve state of pluripotency, which characterizes the early epiblast of the pre-implantation embryo. EpiSCs represent a lesser state of pluripotency, referred to as the primed state, which characterizes the late epiblast of the early post-implantation embryo (Nichols and Smith, 2009).

All ESCs and iPSCs generated so far from other mammalian species than rodent, including primates and rabbit, resemble mouse EpiSCs, and display most of the features of primed pluripotency (Savatier *et al.*, 2017). The generation of human and rabbit PSCs in the naïve state of pluripotency would considerably facilitate their application in medicine and biotechnology, respectively. In addition to their common PSC characteristics, rabbits and humans also share comparable immunology mechanisms, identical lipid physiology, the same early embryonic development and a similar placentation system. These reasons explain why the laboratory rabbit (*Oryctolagus cuniculus*) is widely used as a model for human diseases, and is a more relevant model than the mouse for a wide range of human pathophysiologic disorders (Duranthon *et al.*, 2012). Additive transgenesis of normal or mutant human genes by DNA microinjection into zygotes have been used to create rabbit models for more than thirty years (Houdebine, 2007). Despite its low efficacy, this method has allowed the creation of rabbit bioreactors for producing human therapeutic proteins in their milk (Houdebine, 2009). Recently, the rapid expansion of transgenesis

methods using endonucleases, such as zinc finger nucleases (ZFNs) (Flisikowska *et al.,* 2011), transcription activator-like effector nucleases (TALENs) (Song *et al.,* 2013) and especially clustered regularly interspaced short palindromic repeats/crispr associated Protein 9 (Crispr/Cas9) (Yang *et al.,* 2019), have considerably increased the number of knock-out rabbit models. However, it is much more difficult to apply these technics directly in embryos to precisely target the addition, replacement or mutation of genes. For these purposes, naïve rbPSCs capable of producing germline chimaera would be very useful. In addition, rabbit iPSCs (rbiPSCs) could be used in species preservation and biodiversity maintenance, by allowing the cryopreservation of somatic tissues easily collected on the field without any animal sacrifice. As shown previously with mouse iPSCs, reprogrammed cells could be differentiated into functional male (Cai *et al.,* 2013) or female (Hayashi and Saitou, 2013) gametes, used as nucleus donor cells for nuclear transfer cloning (Yang *et al.,* 2007), or microinjected into host embryos to produce germline chimaera (Bradley *et al.,* 1984). Finally, naïve rbPSCs would be also interesting for the production of organoids, as these new tools allow numerous functional or toxicology studies *in vitro*, and represent indispensable complement to animal models (Sun and Ding, 2017).

Rabbit ESCs (rbESCs) were first derived from early blastocysts (E3.5-E4.0) and cultured on feeder cells by Wang *et al.* (2007). They form flat colonies, self-renew under the dependency of the Activine and FGF2 pathways, fail to colonize rabbit host embryos (Osteil *et al.*, 2013), and are therefore in the primed state of pluripotency. Attempts to derive naïve rbESCs using protocols originally developed in rodents and humans have been unsuccessful (Osteil *et al.,* 2016). The case of rbiPSCs seems more promising, as they exhibit some differences with rbESCs pertaining to their pluripotency state (Osteil *et al.,* 2013). They were produced by reprogramming rabbit somatic cells using the classical combination of the four human genes (*OCT4, SOX2, KLF4, c-MYC*) overexpressed by retroviral vectors (Honda *et al.*, 2010). They display the same morphology as rbESCs and self-renew with FGF2 and Activin, but they are more resistant to single-cell dissociation and perform better in a clonogenic self-renewal assay than do rbESCs. In addition, rbiPSCs exhibit a global gene expression profile closer to that of rabbit epiblast cells and are able to produce chimeric blastocysts by aggregation with morula cells. Attempts to reprogram rbiPSCs toward the naïve state exploiting direct or indirect overexpression of KLF2 and KLF4 factors gave interesting results. Lines obtained with Forskolin to reinforce expression of KLFs formed dome-shaped colonies and showed a reactivation of the second X-chromosome in female rbiPSC lines (Jiang *et al.*, 2014). Other lines overexpressing KLFs exhibited a reconfiguration of both transcriptome and epigenome and improved chimeric competence compared to conventional rbiPSCs (Tapponnier *et al.*, 2017).

In order to use rbPSCs as biotechnology tools and notably for species preservation (Afanassieff et al., 2019), pluripotency characteristics of cells should be maintained after cryopreservation in a medium without any animal-derived products to avoid health risk, and with low concentration of cryoprotectant to reduce toxic risk. To achieve this goal, we have developed a slow-freezing protocol based on a commercial chemically-define media, the CRYO3 (Ref 5617, Stem Alpha, France), with low concentration of dimethyl sulfoxide (DMSO) as cryoprotectant. The CRYO3 was initially designed for clinical applications to replace serum in the freezing medium of human somatic and adult stem cells (Jurga *et al.*, 2012), and has already demonstrated its effectiveness in cryopreserving rabbit embryos (Bruyere et al., 2013).

MATERIALS AND METHODS

Cells and experimental design

RbESCs (Osteil *et al.*, 2016) and rbiPSCs (Osteil *et al.*, 2013) were cultured as described previously. After dissociation and centrifugation, $2x10^6$ cells were resuspended in 1mL of three different media, CRYO3, foetal bovine serum (FBS) for rbESCs and FBS + knock-out serum replacement (KOSR) for rbiPSCs, containing 0, 2.5, 3, 4, 5 or 10% of DMSO, and transferred into cryotubes. Cryotubes were placed into a freezing container (Nalgene, USA), disposed in a -80°C freezer for 15h and then plunged into liquid nitrogen for storage during at least 2 weeks. The cooling rates, calculated using a thermocouple within the cryotube in the presence of cells and freezing media, were on average 0.8°C/min in the linear part of the curves.

Cells analysis after thawing

Cryovials were thawed at 37°C directly from the liquid nitrogen to a water bath, and cells were diluted by addition of 6mL of 37°C culture media drop by drop. After centrifugation and resuspension, 1/5 of the cell

suspensions were used for mortality assessment by flow cytometry and 4/5 were cultured for growth recovery during 3 weeks and analysis of pluripotency gene expression (*OCT4, NANOG, REX1, ESRRB, CDH1, CDH2*) by RT-qPCR at passages 1 and 6.

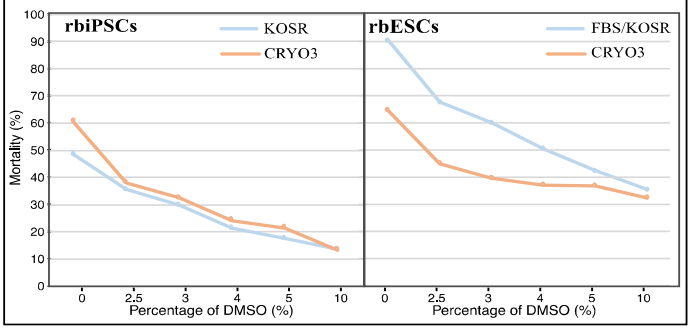
Statistical Analysis

Statistical analyses were performed on R software. The results correspond to 3 replicates for each condition and are presented as means. Mortality, growth recovery and gene expression were analyzed using a linear model. The medium and the DMSO concentration were included in the models as fixed effects. In order to determine the best fit model, AICc were performed. A Tukey adjustment was used to compare each condition at a fixed 5 % error level. Differences with $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Effect of freezing media and DMSO concentrations on rbPSC mortality

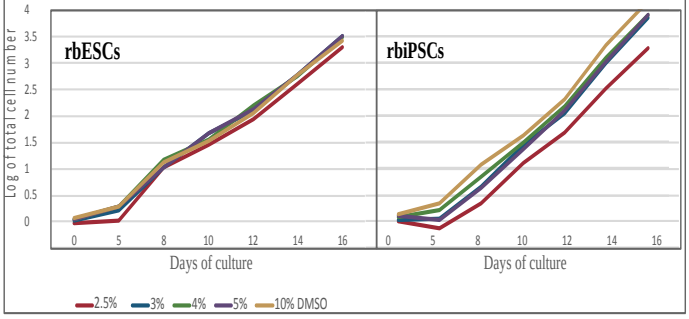
Figure 1: Mortality of rbPSCs after thawing



Before freezing, mortality rates of rbPSCs placed in single-cell suspension were around 10%. After freezing in CRYO3- or FBS/KOSR-based media, the mortality of rbPSCs increased along with the reduction in DMSO concentration. Significant effects of media and DMSO percentages on cell mortality were observed, particularly for the rbESCs, which had lower mortality in CRYO3 with at least 3% DMSO. Thus, it appears that freezing in CRYO3 is already effective with 3% DMSO.

Effect of freezing media and DMSO concentrations on rbPSC growth

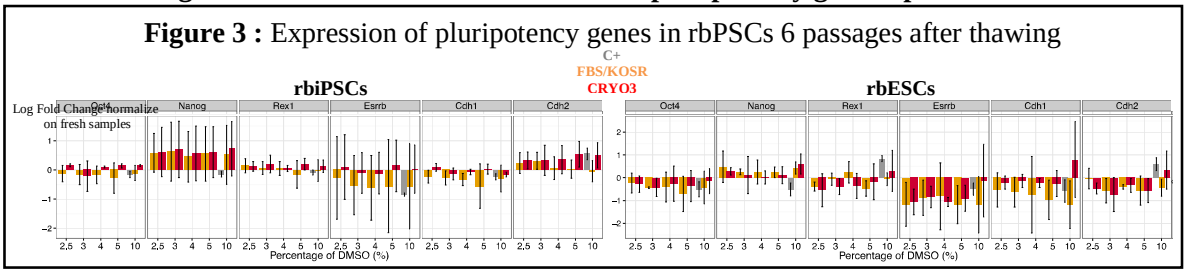
Figure 2 : Growth of rbPSCs after freezing in CRYO3



All the fixed parameters (freezing medium and DMSO concentration), besides the time, influenced significantly the total number of recovered cells. However, the observed differences are slight and similar growth patterns were obtained between the freezing media and the DMSO concentrations. Generally, no significant difference in rbESC and rbiPSC growth recoveries was found between CRYO3- or FBS/KOSR-based media containing 4, 5 and 10 % DMSO.

Effect of freezing media and DMSO concentrations on pluripotency gene expression of rbPSCs

Figure 3 : Expression of pluripotency genes in rbPSCs 6 passages after thawing



Expression profiles of pluripotency genes were affected by freezing of rbPSCs, showing most often down-regulation during the first passage after thawing (data not shown). However, after recovery of the cells at

passage 6 (Figure 3), these expression patterns were closer to those of the fresh samples used for data normalization. In particular, we observed that at passage 6, gene expressions in frozen samples with 4% and 5% DMSO were mainly closer to those in fresh samples, compared to frozen samples with 10% DMSO.

CONCLUSIONS

In this study, we showed that CRYO3-based medium can replace FBS/KOSR-based medium for freezing rbPSCs, especially if the DMSO concentration is reduced to 4-5%. Indeed, this synthetic medium induces acceptable mortality rates, normal growth recoveries, and typical levels of pluripotency gene expression in the presence of only 4% or 5% cryoprotectant. These results are relevant for cryopreservation of pluripotent stem cells, because the conventional concentration of DMSO used is 10%, even in a commercial ready-to-use freezing medium such as the CRYOStor® commonly employed by the stem cell research community.

ACKNOWLEDGEMENTS

This work was essentially supported by grants from Infrastructure Nationale en Biologie et Santé CRB-Anim (ANR-11-INBS-0003), Laboratoire d'Excellence Revive (ANR-10-LABX-73) and INRAE.

REFERENCES

Afanassieff M, Gavin-Plagne L, Perold F, Voisin S, Moreira S, Buff S, Savatier P, Joly T. 2019. Interest of rabbit induced pluripotent stem cells for species preservation. *Thaï Veterinary Medecine Journal, 49 (Suppl. 2), 103-107.*

Bradley A, Evans M, Kaufman MH, Robertson E. 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature, 309, 255-256.*

Bruyere P, Baudot A, Joly T, Commin L, Pillet E, Guerin P, Louis G, Josson-Schramme A, Buff S. 2013. A chemically defined medium for rabbit embryo cryopreservation. *PloS One, 8, e71547.*

Cai H, Xia X, Wang L, Liu Y, He Z, Guo Q, Xu C. 2013. In vitro and in vivo differentiation of induced pluripotent stem cells into male germ cells. *Biochem Biophys Res Commun, 433, 286-291.*

Duranthon V, Beaujean N, Brunner M, Odening KE, Santos AN, Kacskovics I, Hiripi L, et al. 2012. On the emerging role of rabbit as human disease model and the instrumental role of novel transgenic tools. *Transgenic research, 21, 699-713.*

Flisikowska T, Thorey IS, Offner S, Ros F, Lifke V, Zeitler B, Rottmann O, Vincent A, Zhang L, Jenkins S et al. 2011. Efficient immunoglobulin gene disruption and targeted replacement in rabbit using zinc finger nucleases. *PloS One, 6, e21045.*

Hayashi K, Saitou M. 2013. Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. *Nature protocols, 8, 1513-1524.*

Honda A, Hirose M, Hatori M, Matoba S, Miyoshi H, Inoue K, Ogura A. 2010. Generation of induced pluripotent stem cells in rabbits: potential experimental models for human regenerative medicine. *J Biol Chem, 285, 31362-31369.*

Houdebine LM. 2007. Transgenic animal models in biomedical research. *Methods Mol Biol, 360, 163-202.*

Houdebine LM. 2009. Production of pharmaceutical proteins by transgenic animals. *Comp Immunol Micro Infect Dis, 32, 107-121.*

Jiang Y, Kou Z, Wu T, An W, Zhou R, Wang H, Gao Y, Gao S. 2014. Xist deficiency and disorders of X-inactivation in rabbit embryonic stem cells can be rescued by transcription-factor-mediated conversion. *Stem Cells Dev, 23, 2283-2296.*

Jurga M, Forraz N, Basford C, Atzeni G, Trevelyan AJ, Habibollah S, Ali H, et al. 2012. Neurogenic properties and a clinical relevance of multipotent stem cells derived from cord blood samples stored in the biobanks. *Stem Cells Dev, 21, 923-936.*

Nichols J, Smith A. 2009. Naive and primed pluripotent states. *Cell stem cell, 4, 487-492.*

Osteil P, Moulin A, Santamaria C, Joly T, Jouneau L, Aubry M, Tapponnier Y, Archilla C, et al. 2016. A Panel of Embryonic Stem Cell Lines Reveals the Variety and Dynamic of Pluripotent States in Rabbits. *Stem Cell Reports, 7, 383-398.*

Osteil P, Tapponnier Y, Markossian S, Godet M, Schmaltz-Panneau B, Jouneau L, Cabau C, Joly T, et al. 2013. Induced pluripotent stem cells derived from rabbits exhibit some characteristics of naive pluripotency. *Biology open, 2, 613-628.*

Savatier P, Osteil P, Tam P. 2017 Pluripotency of embryo-derived stem cells from rodents, lagomorphs, and primates: Slippery slope, terrace and cliff. *Stem Cell Res, 19, 104-112.*

Sun Y, Ding Q. 2017. Genome engineering of stem cells organoids for disease modeling. *Protein Cell, 8, 315-327.*

Song J, Zhong J, Guo X, Chen Y, Zou Q, Huang J, Li X, Zhang Q, Jiang Z, Tang C et al. 2013. Generation of RAG 1- and 2-deficient rabbits by embryo microinjection of TALENs. *Cell Res, 23, 1059-1062.*

Tapponnier Y, Afanassieff M, Aksoy I, Aubry M, Moulin A, Medjani L, et al. 2017. Reprogramming of rabbit induced pluripotent stem cells toward epiblast and chimeric competency using Kruppel-like factors. *Stem Cell Res, 24, 106-117.*

Wang S, Tang X, Niu Y, Chen H, Li B, Li T, Zhang X, Hu Z, Zhou Q, Ji W. 2007. Generation and characterization of rabbit embryonic stem cells. *Stem cells, 25, 481-489.*

Yang D, Xu J, Chen YE. 2019. Generation of Rabbit Models by Gene Editing Nucleases. *Methods Mol Biol, 1874, 327-345.*

Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T. 2007. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nat Genet, 39, 295-302.*