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Reprogramming of rabbit induced pluripotent stem cells toward epiblast and chimeric competency using Krüppel-like factors

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ABSTRACT

Rabbit induced pluripotent stem cells (rbiPSCs) possess the characteristic features of primed pluripotency as defined in rodents and primates. In the present study, we reprogrammed rbiPSCs using human Krüppel-like factors (KLFs) 2 and 4 and cultured them in a medium supplemented with fetal calf serum and leukemia inhibitory factor. These cells (designated rbEKA) were propagated by enzymatic dissociation for at least 30 passages, during which they maintained a normal karyotype. This new culturing protocol resulted in transcriptional and epigenetic reconfiguration, as substantiated by the expression of transcription factors and the presence of histone modifications associated with naïve pluripotency. Furthermore, microarray analysis of rbiPSCs, rbEKA cells, rabbit ICM cells, and rabbit epiblast showed that the global gene expression profile of the reprogrammed rbiPSCs was more similar to that of rabbit ICM and epiblast cells. Injection of rbEKA cells into 8-cell stage rabbit embryos resulted in extensive colonization of ICM in 9% early-blastocysts (E3.5), epiblast in 10% mid-blastocysts (E4.5), and embryonic disk in 1.4% pre-gastrulae (E6). Thus, these results indicate that KLF2 and KLF4 triggered the conversion of rbiPSCs into epiblast-like, embryo colonization-competent PSCs. Our results highlight some of the requirements to achieve bona fide chimeric competency.

1. Introduction

Pluripotent stem cells (PSCs) exist in two distinct states: naïve or ground state of pluripotency (e.g., mouse embryonic stem cells [mESCs] generated from E4.5 epiblast of blastocyst (Boroviak et al., 2014)), and primed state of pluripotency e.g., mouse epiblast stem cells [mEpiSCs] generated from E6–E7.5 epiblast of early post-implantation embryos (Brons et al., 2007; Nichols and Smith, 2009; Tesar et al., 2007). mESCs and mEpiSCs show many differences, both in terms of molecular characteristics and functional properties (Nichols and Smith, 2009). In particular, mEpiSCs, unlike mESCs, cannot create somatic and germ line chimeras after injection into blastocysts (Brons et al., 2007; Tesar et al., 2007). mEpiSCs can be converted to mESCs by enforced expression of genes such as *Klf2*, *Klf4*, *Nanog*, *STAT3* and *Nr5a2* (Guo and Smith, 2010; Guo et al., 2009) (Hall et al., 2009; Silva et al., 2009; Yang et al., 2010), or by inhibiting CK1, MEK and SMAD2 activity and enhancing WNT activity (Illich et al., 2016). The reverted ESCs acquired the characteristics of naïve pluripotency, including transcriptome and epigenome reconfiguration and, when tested, the capacity to make germline chimeras.

PSCs derived from humans and non-human primates (NHPs) exhibit most of the defining features of primed pluripotency (Nichols and Smith, 2009; Vallier et al., 2005; Wianny et al., 2008), including the

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inability to colonize pre-implantation embryos and produce somatic chimeras (Tachibana et al., 2012). Induced pluripotent stem cells (iPSCs) derived from humans and NHPs also exhibit primed pluripotency-like characteristics. Several studies have reported on the reversion of conventional human ESCs and iPSCs to naïve-like pluripotency using different combinations of transcription factors (KLF2, NANOG, and STAT3), growth factors (LIF, Activin A, and FGF2), and chemical inhibitors of various kinases (ROCK, BRAF, $p38^{MAPK}$, GSK3, MEK1/2, SRC, and JNK). The reverted cells, known variously as NHSM (Gafni et al., 2013), 3iL (Chan et al., 2013), Reset (Takashima et al., 2014), 6i/L/A (Theunissen et al., 2014) and TL2i (Chen et al., 2015a), display some characteristic features of naïve pluripotent rodent stem cells, including reconfigured transcriptome and epigenome, alterations in mitochondrial respiration (Takashima et al., 2014), loss of FGF2 and ERK dependency (Chen et al., 2015a; Takashima et al., 2014), and gain of LIF/STAT3 dependency (Chan et al., 2013; Chen et al., 2015a; Takashima et al., 2014). Reversion of cynomolgus macaque ESCs to naïve-like pluripotency has also been reported (Chen et al., 2015b). When introduced into host macaque embryos, these reverted cells seem capable of colonizing the epiblast and differentiating into cells characteristic of the three germ layers in the fetus. However, the frequency of chimerism is low, possibly because very few cells have truly acquired a naïve state. It is also possible that these rare cells have a weak competitive edge against the host cells in contributing to tissue differentiation.

Rabbit (*Oryctolagus cuniculus*) PSCs exhibit the characteristic features of primed pluripotency. They are dependent on FGF2 signaling and TGFβ family receptors for self-renewal (Honda et al., 2009; Osteil et al., 2013; Wang et al., 2008; Wang et al., 2006). Previously, we have shown that they also lack chimeric competency (Osteil et al., 2016; Osteil et al., 2013). In one of those studies, we compared rbESCs with rabbit iPSCs (rbiPSCs) and showed that rbiPSCs spontaneously exhibited some features of naïve pluripotency, including the activity of the distal enhancer of mouse *Oct4*, permissiveness to single-cell dissociation, and high clonogenicity (Osteil et al., 2013).

In the present study, we explored the conditions for reprogramming rbiPSCs into PSCs with molecular and functional properties more akin to the rabbit epiblast. To that end, we overexpressed both KLF2 and KLF4 in the rbiPSCs, and subsequently adapted the cells to propagation in a medium supplemented with LIF and serum. We then explored the transcriptomic and epigenomic reconfiguration, and asked whether the reprogrammed cells acquired the capability to participate in the formation and expansion of the rabbit epiblast.

2. Material and methods

Full details of the experimental procedure are included in Supplemental Experimental Procedures.

2.1. Reprogramming of B19-EOS iPS cells with human Klf2 and Klf4

B19-EOS cells were produced by somatic cell reprogramming of adult rabbit fibroblasts with the human transcription factors OCT4, SOX2, c-MYC, and KLF4, followed by infection with a lentiviral vector, L-SIN-EOS-C(3)-EiP (EOS) (Hotta et al., 2009), and subsequent selection of EOS-expressing cells using puromycin (Osteil et al., 2013). In the *EOS* vector, the GFP and puromycin resistance genes are under the transcriptional control of a minimal early transposon promoter and trimer of CR4 enhancer of mouse *Oct4*. The CR4 enhancer overlaps a distal enhancer whose activity is associated with naïve-like pluripotency (Yeom et al., 1996). In the present study, the B19-EOS cells were transfected with the *pGG137-hK2-2A-hK4* vector, which is a PiggyBac vector expressing human *KLF2* and *KLF4*. Three clones showing low, medium, and high expression of KLF2 and KLF4 were selected. These clones were designated as rabbit Enhanced KLF Activity (rbEKA)-low (low KLF expressor), rbEKA-mid (mid KLF expressor), and rbEKA-high (high KLF expressor).

2.2. Isolation of ICM and epiblast

Early-blastocysts (E4) were collected 96 h after artificial insemination and incubated in 5 mg/ml pronase at room temperature to remove the zona pellucida and mucin coat. The ICM was separated from the trophectoderm by immunosurgery followed by gentle pipetting with a glass pipette. To prepare the epiblasts, expanded blastocysts (E6) were collected 147 h after artificial insemination and placed in FHM medium (Millipore). The zona pellucida was mechanically removed. The embryo was opened and flattened on a plastic dish to expose the embryoblast. The hypoblast was first dissociated by careful scratching with a glass needle, and the epiblast was then separated from the trophoblast with a microscalpel.

2.3. Microarray analysis

We used a customized rabbit microarray containing 62,976 probes (platform GPL16709) (Jacquier et al., 2015). Forty ICMs and 10 epiblasts were pooled prior to RNA extraction. For hybridization, cyanine-3 (Cy3)-labeled cRNAs were prepared from 20 ng RNA (ICM and epiblast samples) and 200 ng RNA (cell lines) by using One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labelling Kit (Agilent Technologies). To verify that the use of a 10-fold lower quantity of RNA from embryo samples as compared with cell lines, did not significantly skew the outcome of the hybridization experiment, test hybridization using 20 ng and 200 ng of RNA from cell lines was performed, and the intensity of the hybridization signals was compared between both conditions. None of the 62,976 probes showed a statistically significant difference in signal intensity between the two RNA quantities (BH corrected p-value < 0.05, ratio > 2), validating our experimental paradigm. For all samples, 0.6 μg Cy3-labeled cRNA (specific activity, >6.0 pmol Cy3/μg of cRNA) was used for hybridization of the microarray. After hybridization, the scanned images were analyzed using Feature Extraction Software (7.10.3.1; Agilent Technologies). Data were normalized using intra-array median subtraction and $\log_2$ transformation. Differential analysis was performed using Limma R package (http://www.r-project.org), and JADE R package was used to compute independent component analyses (ICA).

2.4. Single-cell gene expression analysis with qRT-PCR

Real-time PCR (qPCR) was performed using the CellsDirect™ One-Step qRT-PCR kit (ThermoFisher) coupled with the StepOnePlus real-time PCR system and Fast SBYR® Green Master Mix (Applied Biosystems). Expression of the target genes was normalized to that of the rabbit TATA-box binding protein (*Tbp*) and *Gapdh* genes. For single-cell qPCR, cells were dissociated using trypsin, manually separated. Reverse transcription and specific target amplification were performed using the SuperScript III/RT Platinum Taq mix provided in the kit. The pre-amplified products were subsequently analyzed with Universal PCR TaqMan Master Mix (Applied Biosystems) and coupled with a DNA Binding Dye Sample Loading Reagent (Fluidigm) and Evagreen (Biotium 31000) in 96.96 Dynamic Arrays on a BioMark System.

2.5. miRNA quantification

Mature miRNA quantification was performed via a two-step protocol including reverse transcription with miRNA-specific primers using a TaqMan MicroRNA Reverse Transcription Kit, followed by quantitative real-time PCR with TaqMan MicroRNA Assays-TM. Expression of the target miRNAs was normalized to the housekeeping miRNAs *ocu-miR-191* and *ocu-miR-423*.