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Review

# Generation of rabbit pluripotent stem cell lines

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**Abstract**

Pluripotent stem cells have the capacity to divide indefinitely and to differentiate into all somatic cells and tissue lines. They can be genetically manipulated in vitro by knocking genes in or out, and therefore serve as an excellent tool for gene function studies and for the generation of models for some human diseases. Since 1981, when the first mouse embryonic stem cell (ESC) line was generated, many attempts have been made to generate pluripotent stem cell lines from other species. Comparative characterization of ESCs from different species would help us to understand differences and similarities in the signaling pathways involved in the maintenance of pluripotency and the initiation of differentiation, and would reveal whether the fundamental mechanism controlling self-renewal of pluripotent cells is conserved across different species. This report gives an overview of research into embryonic and induced pluripotent stem cells in the rabbit, an important nonrodent species with considerable merits as an animal model for specific diseases. A number of putative rabbit ESC and induced pluripotent stem cell lines have been described. All of them expressed stem cell-associated markers and maintained apparent pluripotency during multiple passages in vitro, but none have been convincingly proven to be fully pluripotent in vivo. Moreover, as in other domestic species, the markers currently used to characterize the putative rabbit ESCs are suboptimal because recent studies have revealed that they are not always specific to the pluripotent inner cell mass. Future validation of rabbit pluripotent stem cells would benefit greatly from a validated panel of molecular markers specific to pluripotent cells of the developing rabbit embryos. Using rabbit-specific pluripotency genes may improve the efficiency of somatic cell reprogramming for generating induced pluripotent stem cells and thereby overcome some of the challenges limiting the potential of this technology.


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**Contents**



## 1. Introduction

The rabbit is one of the most frequently used and relevant nonrodent species for modeling disease processes. The main biomedical applications of genetically modified organism rabbits are as models for human disease and in biopharming (live bioreactors) for the large scale production of pharmaceutically important proteins required for the treatment of human diseases.

Recombinant protein expression in the milk of genetically modified organism animals has been extensively studied in the past 25 yr, and has undergone significant recent improvements both from a methodological point of view and in terms of reaching the market. In 2010, Pharming Group NV (Leiden, The Netherlands) received European approval for a product (Ruconest) for treating acute attacks of hereditary angioedema; the drug which is now on the market is harvested from the milk of transgenic rabbits.

The rabbit has an advantage over rodents in human disease modeling in that it is phylogenetically closer to primates than the rodents [1] and large enough to permit nonlethal monitoring of physiological changes. For these reasons, a number of research groups have chosen transgenic rabbits as models to study lipoprotein metabolism and atherosclerosis. In this respect, the economically most important chronic diseases of the metabolic syndrome complex: diabetes mellitus, hypertension, atherosclerosis, hyperlipidemia, and obesity cannot be adequately mimicked in mice. The rabbit is also a more appropriate model than rodents for prenatal development and the long-lasting effects of perturbations in the prenatal period on adult health and complex diseases. Similarities in biochemical and physiological processes, including placental structure, between human and rabbit make the latter an excellent model for reproductive studies [2,3]. In addition, because rabbits have relatively large eyes, studies on the pathophysiology of retinal degeneration using the rabbit as a model have recently been published [4]. Beyond that, transgenic rabbit models of human disorders connected to cardiac electrophysiology and hypertrophy have turned out to be extremely useful in pharmacogenomic studies and design of disease prevention programs [5].

## 2. Biology of pluripotency in rabbit

### *2.1. Stem cell regulatory pathways in rabbits*

Pluripotency and the capacity for self-renewal allow embryonic stem cells (ESCs) to either divide continuously in the undifferentiated state or to differentiate into any somatic cell type. These characteristics are controlled by a number of cell signaling pathways and therefore depend on the provision of specific conditions or factors. Rabbit embryonic stem cells (rESC) resemble primate ESCs more closely than mouse ESCs [6]. The most important signaling pathways involved in the maintenance of pluripotency and the capacity for self-

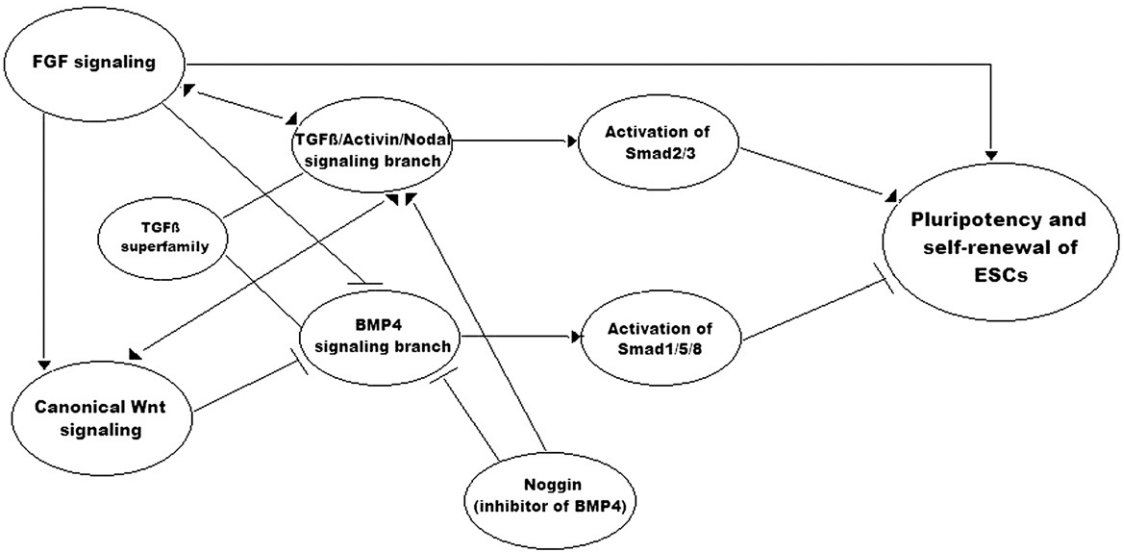


Fig. 1. A scheme illustrating the interactions among fibroblast growth factor (FGF), transforming growth factor (TGF), bone morphogenetic protein (BMP), and Wnt signaling to sustain rabbit embryonic stem cells (rESC) self-renewal (based on Wang et al. 2008 [6]).

renewal in primate ESCs are fibroblast growth factor (FGF), which activates the mitogen-activated protein kinase and Akt pathways and transforming growth factor (TGF)-$\beta$ which acts through Smad2/3/4. The Wnt pathway also supports pluripotency by activating $\beta$-catenin. Signaling through these pathways results in the activation and expression of three essential pluripotency-associated transcription factors: Oct4, Sox2, and Nanog. These factors are all very important in maintaining the pluripotent state of ESCs. The interaction between Sox2 and Oct4 activates expression of pluripotent-specific target genes and also regulates their own expression. Nanog is also incorporated into the Sox2-Oct4 complex, which is a transcription factor with an essential role in maintaining the pluripotent state of the inner cell mass (ICM) and of ESCs derived from the ICM [7]. Nanog suppresses differentiation of ESCs toward the extraembryonic endoderm and trophectoderm lineages. In interaction with Smad1, Nanog inhibits bone morphogenetic protein (BMP)-induced mesoderm differentiation of ESCs.

However, the signaling pathways that regulate ESC pluripotency in the rabbit have not been definitively identified. Wang et al. showed that inhibition of the TGF, FGF, and canonical Wnt/catenin (Wnt) pathways resulted in the differentiation of rESC followed by downregulation of Smad2/3 phosphorylation and $\beta$-catenin expression and upregulation of the phosphorylation of Smad1 and catenin. Together the FGF and TGF pathways play important roles in the maintenance of pluripotency by rESCs and in the activation of Smad2/3 and inhibition of Smad1/5/8 signaling. FGF activity depends on the TGF pathway, although FGF alone also has effects directly or indirectly on the self-renewal of rESCs. Wnt signaling has a less critical role than the FGF and TGF pathways, but also affects rESC state. Wang et al. showed that the TGF, FGF, and Wnt pathways are required for rESC pluripotency and that inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/AK mouse strain thymoma (AKT) signaling facilitates rESC differentiation [6] (Fig.1).

### 2.2. *Rabbit-specific pluripotency gene sequences*

ESCs and induced pluripotent stem cells (iPSCs) are pluripotent cells with the ability to differentiate into components of all three embryonic germ layers (ectoderm, endoderm, and mesoderm). There are a small number of transcriptional regulators, including Oct4, Sox2, and Nanog, that are central components of this pluripotency network. This network contributes to self-renewal and prevents differentiation. While promoting the equipoised state of pluripotency, these factors also control differentiation toward specific cell lineages [8–11]. Genes involved in maintaining the pluripotent cell state have been well characterized functionally in the mouse. However, little is known about the regulation of self-renewal and pluripotency in rESCs and rabbit iPSCs (riPSCs).

The importance of the pluripotency genes was clearly demonstrated when, in 2006, Takahashi and Yamanaka [12] identified four factors which, when cotransfected and expressed in mouse adult fibroblast cells, caused those cells to revert to the pluripotent state. These four factors were:

1. POU (**P**ituitary-specific Pit-1, **O**ctamer transcription factor proteins Oct-1 and Oct-2, neural **U**nc-86 transcription factor from Caenorhabditis elegans) class 5 homeobox (1Oct4/Pou5f1) encoded by the gene pou5f1 (POU domain, class 5, transcription factor 1); a transcription factor expressed in undifferentiated ESCs. Oct4 expression is critical to the maintenance of the pluripotent state.
2. SRY (sex-determining region Y)-box containing gene 2 (Sox2) is another transcription factor critical for the maintenance of pluripotency. It acts in parallel with Oct4 in regulating the expression of target genes involved in the maintenance of pluripotency.
3. v-myc myelocytomatosis viral oncogene homolog (c-Myc) is a well known proto-oncogene. This gene encodes a transcription factor that controls the expression of genes involved in the regulation of cell proliferation, growth, differentiation, apoptosis, and malignant transformation.
4. Krüppel-like factor 4 (Klf-4) is a transcription factor expressed in undifferentiated ESCs. It is also expressed in specific cells in the adult organism, including cells in the gut, testis, and lungs. It regulates proliferation, differentiation, and cell survival.

Although Nanog does not belong to the original four "Yamanaka factors", is also an important pluripotency factor that acts together with Oct4 and Sox2 in establishing ESC identity. Besides this, Nanog is downstream of Klf4 and is switched on by the four factors during iPSC reprogramming.

One approach to identifying putative rabbit pluripotency genes is based on phylogenetic sequence comparison among species. Using basic local alignment search tool (BLAST) searches against public databases, the homologues of several mammalian pluripotency genes (*Nanog, Oct4, Klf4, c-Myc, Sox2*) have been identified. Another approach to identifying candidate pluripotency factors is to perform a search in the Rabbit Genome Resource Database, because the sequencing of the rabbit genome is nearly complete. These databases have yielded partial and/or full rabbit *Nanog, Oct4, c-Myc*, and *Sox2* sequences. The gene sequences found in the Rabbit Genome Resource Database show different degrees of homology to the mouse and human orthologs. Those for Oct4 and c-Myc are relatively close, while Nanog differs more at both the nucleic and amino acid levels [13]. The identified differences might explain the difficulties encountered using human or murine cytokines to establish rESC and riPSC that are capable of germ line transmission via chimera production.

### 2.3. MicroRNA regulation of stemness in the rabbit

Recent studies have indicated that microRNAs (miRNAs), a class of noncoding endogenous small RNA molecules, participate in posttranscriptional regulation of gene expression and play a key role in stem cell self-renewal and differentiation [14]. ESC-specific miRNAs are abundant in pluripotent stem cells, but disappear rapidly during differentiation [14,15]. The miR290-295 cluster is expressed during early embryogenesis and is functionally important in mouse ESCs and embryonic carcinoma cells [14]. Overexpression of miR290-295 prevents ESCs from embarking on differentiation, probably by preventing ESCs accumulating in the G1 phase [16]. The miR302-367 cluster is also highly expressed in both human and murine ESCs, and downregulated upon differentiation [17]. In addition, DGCR8 and Dicer knockout studies indicate that miRNA pathways have a key regulatory function in ESC self-renewal and pluripotency [18]. Moreover, the ESC-specific miRNA promoters are regulated by Oct4, Sox2, and Nanog [19–22]. The ESC-specific miRNAs can directly target these transcription factors, establishing a complex feedback loop, which is a key regulator of pluripotency [22]. A recent study on iPSC derivation shows that expression of the miR302-367 cluster rapidly and efficiently reprograms mouse and human somatic cells to an iPSC state without the requirement for exogenous transcription factors, and that it is even more efficient than the standard Oct4/Sox2/Klf4/Myc-mediated method [23]. Other reports show that c-Myc replacement by miR-294 can enhance the reprogramming of somatic cells to iPSCs [24]. Based on these studies, we aimed to characterize rabbit stem-like cell specific miRNAs. We observed high expression of miR302a, miR302b, and miR367, whereas miR-290, miR-292, and miR-294 were expressed at lower levels in rESCs. We could not detect the expression of the miR371-373 cluster in rabbit ESCs. The SOLiD Sequencing System allowed us to identify numerous miRNAs that appear to be specific to rESCs. The newly described miRNAs

have already been validated on rabbit embryos and ESCs (Maraghechi et al., manuscript in preparation). Further profiling of the rabbit ESC-specific miRNA signature is expected to help solve the problems encountered during rabbit ESC and iPSC derivation.

### 2.4. Antibodies (ABs) which work in the rabbit

Rabbit ESCs and iPSCs morphologically resemble human ESCs; however, their proliferation characteristics more closely resemble those observed for mouse ESCs. Differences were also identified when the cell lines were studied for pluripotency markers by immunocytochemistry. The possible explanations for the differences observed include both technical and biological elements, such as the absence of specific ABs raised against rabbit putative pluripotency protein sequences; and the varying degree of homology between the human and rabbit amino acid sequences used to raise the ABs. The ABs used for characterizing rESCs and riPSCs have all been raised against human protein sequences. This is because the homology between human and rabbit pluripotency marker sequences appears to be high despite the absence of complete, exact rabbit pluripotency gene sequence information. Because it is difficult to generate appropriate rabbit proteins, the use of the commercially available ABs raised against human pluripotency markers is the "next best" option, but there are inevitable failures of cross-reactivity. The most frequently used ABs to detect Oct4 are those produced commercially by Santa Cruz Biotechnology [25–27] and Chemicon [10]. The AB from Santa Cruz Biotechnology is a goat polyclonal AB raised against a peptide mapping near the N-terminus of human Oct4 and that is specific for the nuclear localized Oct4A isoform. This AB exhibits strong positive staining for Oct4 with a nuclear localization. By contrast, the Chemicon AB is an anti-Oct4 mouse monoclonal AB raised against a recombinant protein corresponding to amino acids 143 to 359 of human Oct4 and that detects both nuclear (Oct4A) and cytoplasmic (Oct4B) isoforms, as demonstrated by Wang, et al. [10]. In the case of the stage-specific embryonic antigen-1 (SSEA1), which is one of the most important ESC markers, more contradictory results have been published. The most frequently used ABs for this surface marker are those from Developmental Studies Hybridoma Bank (DSHB) described by Honda et al. [26,28], who generated the first riPSC lines, and that from Chemicon which was used by Wang et al. [10] and by Intawicha et al. [27]. Honda et al. [26,28] and Wang et al. [10] showed potentially positive immunostaining for SSEA1. By contrast, Intawicha et al. [27] did not observe positive staining for SSEA1 using the same AB as Wang et al. [10]. One possible explanation for the inconsistent results might be sample preparation: the presence or absence of detergents like Tween 20 in the blocking solution. Tween 20 is a nonionic detergent that is nonselective in nature and may extract proteins along with the lipids [29–30], making the membranes permeable to ABs. The ABs against the membrane markers SSEA4, Tra-1-60 and Tra-1-81, which are also often used to characterize ESCs and iPSCs, are available both from Chemicon and DSHB and both sets achieve the expected membrane staining. In the case of Nanog, there are two different sets of ABs used regularly: the first one is distributed by Cosmobio, Japan and was used by Honda et al. [26] and the other is provided by Abcam and was used by Intawicha et al. [27]. The latter AB did not work well according to the published data, and since that publication its production has been discontinued.

Thus, based on available publications the ABs that can be used to effectively characterize rESC and riPSCs are those against Oct4 (from Santa Cruz Biotechnology and Chemicon), Nanog (from Cosmobio and Abcam), SSEA1, SSEA4, Tra-1-60, and Tra-1-81 (from Chemicon and DSHB). Overall, this suggests that it would be helpful to produce rabbit-specific ABs raised against rabbit protein sequences, thereby emphasizing the need to clone rabbit specific cDNAs. This would make it feasible to test and produce ABs which work in the rabbit, and could open up further possibilities toward using the rabbit as a model system for human development and disease.

## 3. Technologies to reprogram rabbit somatic cells to pluripotency

### 3.1. Nuclear transfer results and limitations in the rabbit

Reprogramming of somatic cells to pluripotency can be achieved by nuclear transfer (NT) into enucleated oocytes. Compared with normal fertilized preimplantation embryos, somatic cell nuclear transfer (SCNT)-derived embryos have the added challenge of silencing their somatic-specific genes while reactivating all the embryo-related genes and pathways. In this respect, SCNT is a powerful tool for improving our understanding of the signaling pathways involved in pluripotency and differentiation.

In the late 1980s and during the 1990s, the rabbit was one of the mammalian species in which investiga-

tors pioneered nuclear transfer technologies, in particular when the first embryonic clones were produced by introducing rabbit embryonic cell nuclei (blastomeres from 8- to 16-cell stage embryos) into enucleated oocytes [31–34]. Since the first mammals were produced [35] by SCNT, many species have been cloned using this technology. Nevertheless, when differentiated somatic cells were used as donor cells for SCNT in the rabbit, it was found that this species was relatively difficult to clone when compared with other mammals. It was not until 2002 that the first rabbits cloned by SCNT, using freshly prepared adult rabbit cumulus cells as donor cells, were born [36,37]. Since then only a few other groups have published successful generation of cloned live rabbits using somatic donor cells or stem cells [38–41], these did however include transgenic rabbits expressing green fluorescent protein [41,42]. Although a good proportion (up to 55%) of reconstructed embryos develops to the blastocyst stage, the development to term after embryo transfer is low (average 2%). A better development to term rate was obtained when multipotent stem cells were used as donor cells (4%) [41]. Nevertheless, relatively few resulted in live offspring and fewer still survived to sexual maturity; according to published data collected by Zakhartchenko et al. [41], from 42 cloned rabbits born alive, only 17 reached sexual maturity [36–40]. The greatest number of cloned rabbits surviving to adulthood (eight) was reported by Meng et al. [40] using cumulus cells as nuclear donors.

Successful nuclear reprogramming from somatic to pluripotent status is the key event in NT, and depends on the epigenetic status of the nucleus. There is therefore a reason why cells with high genome plasticity potential (mesenchymal stem cells) might be better nuclear donors than more differentiated cell types. In the case of rabbit NT, putative ESCs [25] or iPSCs [26], may be even more successful donor cells for NT. Studies [40,43] have also investigated the use of Trichostatin A (TSA; a histone deacetylase inhibitor) to examine the effect of methylation changes on nuclear reprogramming. In the rabbit, the mechanism and the long-term effects of TSA treatment on postimplantation and postpartum development require further studies, not least because in the studies of Meng et al. [40] all the TSA-treated animals died within 19 days, while among the non–TSA-treated SCNT animals a reasonable proportion survived to adulthood and gave birth to healthy progeny. In the case of the rabbit, progress and difficulties encountered during the SCNT procedure might be informative for identifying the critical factors implicated in reprogramming defects.

### 3.2. Generation of rESC lines

Over the past 5 yr, four independent groups have reported the establishment of rESC (or ESC-like) lines (Table 1A) [10,25,41,44–49]. Rabbit ESCs exhibit the cardinal features of pluripotent stem cells: the capacity to self-renew indefinitely (or at least for a large number of passages) in vitro under appropriate culture conditions, and to differentiate into specialized cells of ectodermal, mesodermal, and endodermal origins. Like their murine and primate counterparts, rESCs also have the capacity to form teratomas when injected into an immunodeficient mouse. One key factor in the derivation of rESC lines appears to be the density of feeder cells [25]. Using the conditions described by Honda and colleagues [26], we could derive several rESC lines. All of them displayed unlimited growth (until passage 50) and ability to differentiate into the three germ layers (Osteil et al. manuscript in preparation). Rabbit ESCs grow in flat colonies, resembling the colonies of primate (human and nonhuman) ESCs. They can be passaged after collagenase treatment followed by gentle dissociation (Osteil et al., unpublished data). It has been shown that both FGF2, through ERK and PI3K activation, and Activin/Nodal signaling, through Smad2/3 activation, are necessary to maintain the pluripotent status of rESCs [6,28]. The question of whether rESCs also require leukemia inhibitory factor (LIF)-signaling for self-renewal is controversial. Studies performed by Honda and colleagues demonstrated that treatment with a janus kinase (JAK) kinase inhibitor, resulting in the loss of phosphorylated signal transducer and activator of transcription-3 (STAT3), had no effect on self-renewal [28]. By contrast, the results of studies performed with ESC lines derived from parthenogenetically activated rabbit embryos suggested that both LIF and FGF2 were capable of sustaining self-renewal, and that the two factors could act cooperatively to support stemness in the absence of feeder cells [27,50]. The rESC lines isolated in our laboratory do not require LIF to ensure a blockade to differentiation. However, the requirement, either of autocrine LIF signaling, or of paracrine LIF signaling involving the feeder cells, acting in synergy with FGF2 signaling cannot be excluded (Osteil et al., unpublished).

In an effort to generate rESCs capable of self-renewal in the naive state of pluripotency, ICM cells were plated on feeder cells in N2/B27 medium supplemented with LIF, ERK (PD03225901), and GSK-3

(CHIR99021) inhibitors (2 inhibitors + LIF medium), according to the protocol described previously for mouse and rat ESC derivation [51,52]. As described for rodents [53], rabbit embryos were also treated with PD0325901 to promote the growth of pluripotent stem cells. Most ICMs from treated and nontreated embryos gave rise to primary outgrowths when plated. No difference was observed between outgrowths originating from PD0325901-treated and control embryos and none of them could be passaged more than once. These results indicate that pluripotent stem cells derived from rabbit ICM are not capable of self-renewal in the culture conditions described previously for rodents.

As indicated above, ingredients of the media for maintaining stable rESC lines differ between laboratories. The basic medium in most cases is Dulbecco's Modified Eagles Medium (DMEM) or supplemented versions thereof, such as DMEM/F12 or Knock-Out DMEM. The common ingredients are glutamine, $\beta$-mercaptoethanol and nonessential amino acids. With regard to the specific supplements, most research groups use either LIF or FGF2, which is why the question of whether rESCs require one or both of these factors is still controversial. The situation is similar with respect to serum content; the medium may contain fetal calf serum or Knock-Out Serum Replacement. To date there is no consensus about what method or solutions are the most effective for the establishment of rESCs.

### 3.3. Generation of riPSC lines

Generation of iPSCs from somatic cells demonstrates that adult mammalian cells can be reprogrammed to a pluripotent state by treating them with four major factors. iPSC technology is a unique tool for deriving specific stem cells to study disease and for developing possible treatments for degenerative disorders. iPSCs can be promising tools for drug screening, toxicology testing, and for generating knockout or transgenic animal models. The first iPSCs were generated by retroviral vector transduction using *c-Myc*, *Klf4*, *Oct4*, and *Sox2* [12,54–56]. The disadvantage of the retroviral system is that it stably integrates into the host genome, causing malignant transformations in chimeric animals [55]. The application of a constitutively active lentivirus system could not overcome this drawback because it was even less efficiently silenced in pluripotent cells than retroviral vectors [57,58]. The next generation of the lentiviral system is represented by the inducible lentiviral vector, in which the expression is regulated by doxycycline. Drug resistance influences the expression of the transgene, reducing the risk of constitutive expression, and providing a selection tool for reprogrammed iPSCs [57,59]. Lentiviral vectors have become more efficient than retroviral vectors at infecting different somatic cell types and can be used to express polycistronic cassettes encoding all four reprogramming factors [60,61]. The best solution given the requirements of safety for eventual therapy is the generation of integration-free iPSCs. Techniques used to generate integration-free iPSCs can be divided into three categories: (1) methods using nointegrating vectors, (2) methods using integrating vectors that can be removed from the genome, and (3) methods that do not use nucleic acid-based vectors at all. Several laboratories have developed integration-dependent gene delivery vectors containing loxP sites permitting the excision of a transgene by Cre-recombinase [62,63]. For example, PiggyBac [64,65] and Sleeping Beauty transposons [66–68] can be introduced into the host genome and removed by the transposase or a cre-lox system. Successful reprogramming was also achieved by delivering the reprogramming factors as purified recombinant proteins [69], or using synthetic modified messenger RNA (mRNA) [70] and miRNA [23]. In human and murine cells, these technologies already work to some extent. Using a variety of methods, iPSCs have now been derived from several different species: mouse [12,64,65] human [23,63,70–73], rat [74], rhesus monkey [75], dog [76], horse [77], and pig [78]. Honda et al. [26] were the first group to report the establishment of riPSC 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* (Table 1B). The newly generated riPSCs 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, riPSCs rapidly differentiated into ectoderm, mesoderm, and endoderm. In vivo they formed teratomas, but they were unable to form chimeras. Thus, the newly generated rabbit iPSCs fulfilled all requirements of pluripotency, except the chimera test. However, global gene expression analysis revealed slight, but definite differences between ESCs and iPSCs.

We generated riPSC lines by using retroviral vectors to introduce the human reprogramming genes (*c-Myc*, *Klf4*, *Sox2*, and *Oct4*) into adult rabbit ear fibroblasts. The resulting cells exhibited the cardinal

Table 1
Published studies on establishment of pluripotent stem cell lines from rabbit.

| Year | Reference Number | Title | Special supplements in the media | Markers tested | Level of pluripotency | Level of chimerism |
|---|---|---|---|---|---|---|
| A. Embryonic stem cell lines | | | | | | |
| 1993 | [46] | Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos | Murine leukemia inhibitory factor | Desmin | Differentiation in vivo and in vitro, normal karyotype | No chimera formation |
| 1996 | [47] | Pluripotential rabbit embryonic stem (ES) cells are capable of forming overt coat color chimeras following injection into blastocysts | Murine leukemia inhibitory factor | AP | Alkaline phosphatase activity; differentiation in vivo and in vitro | The efficiency of chimera formation was low (5% of live-born), but the degree of chimerism, as assessed by coat color contribution, was high (10% to 50%) |
| 2006 | [44] | Rabbit embryonic stem cell lines derived from fertilized, parthenogenetic or somatic cell nuclear transfer embryos | Human recombinant basic fibroblast growth factor | AP, SSEA-1, SSEA-3, SSEA-4, TRA-1-10, TRA-1-81, Oct-4, EBAF, FGF4, TDGF1, ß-Tubulin, $\alpha$-fetoprotein | Normal karyotype; alkaline phosphatase activity; differentiation in vivo and in vitro, genetic manipulation, production of nuclear transfer rabbit embryos | No chimera formation |
| 2007 | [10] | Generation and characterization of rabbit embryonic stem cells | No supplemented special factor | SSEA-1, SSEA3, SSEA-4, TRA-1-60, TRA-1-81, Oct4, AP, Nanog, SOX2, UTF-1, FGF, WNT, TGF | Normal karyotype; alkaline phosphatase activity; differentiation in vivo and in vitro | No chimera formation |
| 2008 | [48] | Culture and characterization of presumptive embryonic stem cell line isolated from New Zealand White rabbit embryos | Murine leukemia inhibitory factor | SSEA-1, Oct4, AP, SOX2 | Alkaline phosphatase activity; differentiation in vivo and in vitro | No chimera formation |
| 2008 | [49] | Characterization, chromosomal assignment, and role of LIFR in early embryogenesis and stem cell establishment of rabbits | Murine leukemia inhibitory factor, human recombinant basic fibroblast growth factor | Oct4, SSEA-1, Nanog, CD9, AP, gp130 | Alkaline phosphatase activity; differentiation in vivo and in vitro | No chimera formation |
| 2008 | [25] | Stable embryonic stem cell lines in rabbits: potential small animal models for human research | Murine leukemia inhibitory factor; human recombinant basic fibroblast growth factor | Nanog, SSEA-1, SSEA-4, Oct4, SSEA-3, AP | Alkaline phosphatase activity; differentiation in vivo and in vitro, normal karyotype, telomerase activity | The efficiency of chimera formation was high (36% of live-born), however, based on The green fluorescent protein fluorescence detection the embroyonic stem cells did not contribute to any kind of tissues |

(continued on next page)

Table 1
(continued).

| Year | Reference Number | Title | Special supplements in the media | Markers tested | Level of pluripotency | Level of chimerism |
|---|---|---|---|---|---|---|
| 2009 | [27] | Characterization of embryonic stem cell lines derived from New Zealand white rabbit embryos | Murine leukemia inhibitory factor | Sox2, Nanog, Oct-4, SSEA-4, TRA-1-60, TRA-1-81, AP, MAP2, desmin, GATA4, Jak1, Jak2, Jak3, Stat3, LIFR, gp130 | Alkaline phosphatase activity; differentiation in vivo and in vitro | No chimera formation |
| 2011 | [41] | Cell-mediated transgenesis in rabbits: chimeric and nuclear transfer animals | Human leukemia inhibitory factor; human recombinant basic fibroblast growth factor | AP, Oct4, Rex1, Nanog, Terc, nodal, Foxd3, Dppa5, Bmp4, SSEA-1, SSEA-4, TRA-1–60, TRA-1–81 | Alkaline phosphatase activity; differentiation in vivo and in vitro | The single live-born offspring showed evidence of low level coat color chimerism |
| B. Induced pluripotent stem cell lines | | | | | | |
| 2010 | [26] | Generation of induced pluripotent stem cells in rabbits: potential experimental models for human regenerative medicine | For culture of adult rabbit somatic cells: hepatocyte growth factor, epidermal growth factor. For iPSC induction and culture:murine leukemia inhibitory factor; human recombinant basic fibroblast growth factor | AP, SSEA-1, SSEA-4, Oct-4, Nanog, SSEA-3 | Telomerase activity, normal karyotype, differentiation in vivo and in vitro, alkaline phosphatase activity | No chimera formation |

AP, alkaline phosphatase; iPSC, induced pluripotent stem cell; SSEA, stage-specific embryonic antigen; TRA, tumor rejection antigen; UTF, undifferentiated embryonic cell transcription factor; FGF, fibroblast growth factor; WNT, wingless and integrase-1; TGF, tumor growth factor; gp, glycoprotein; CD, cluster of differentiation; MAP, mitogen activated protein; GATA, globin transcription factor; Dppa, developmental pluripotency associated; FoxD, forkhead box D; Rex1, reduced expression protein; Terc, telomerase RNA component; EBAF, endometrial bleeding-associated factor; TDGF, teratocarcinoma-derived growth factor.

features of nonmurine iPSCs, including the capacity for indefinite self-renewal in the presence of FGF2. Like their embryonic counterpart, riPSCs seem to have a restricted capacity to colonize the preimplantation embryo (i.e., to form chimeras) (Osteil et al., manuscript in preparation).

Recent studies have shown that riPSCs in culture, like human induced pluripotent stem cells, require basic FGF and hypoxic conditions to maintain them in the pluripotent state. While the reprogramming of rabbit somatic cells using human reprogramming factors is not very efficient, we cannot rule out the possibility that using rabbit-specific sequences of the reprogramming factors will improve the efficiency.

### 3.4. Chimera production with ESCs/iPSCs

The first chimeric rabbits were reported in 1974 by Gardner and Munro [79], and this breakthrough was followed by many more attempts. Yang and Foote used cells recovered at stages of development roughly equivalent to the recipient embryo by combining two morulae [80]. In 1980, ICM cells injected into Day-4 blastocysts [81] or fetal gonadal cells [82], or blastomeres from 8- to 16-cell stage embryos [83] injected into other 8- to 16-cell stage embryos resulted in chimeras. The generation of chimeras through blastocyst injection in rodents has been used to generate knockout animals, where gene targeted ESCs are used to transmit a ma-

nipulated genome through the germ line of the chimeric animal. ESC pluripotency, as determined by chimera production after blastocyst injection, has already been published for the mouse [84], and the generation of rabbit chimeras would benefit biomedical research by offering a genetically defined nonrodent model for human diseases.

Honda et al. [26] was the first to report the establishment of riPSCs but could not manage to generate rabbit chimeras from these iPSCs. However, Zakhartchenko and colleagues recently reported one live born ESC-derived rabbit chimera after injection of albino rESCs into 4- to 8-cell stage embryos of the Black Alaska rabbit breed [41]. However, the single live-born offspring, which showed evidence of low level coat color chimerism, died after 2 mo so that inheritance through the germ line could not be tested. Such a low rate of chimera generation may result either from the failure of ESCs to colonize the preimplantation embryo, or from the failure of incorporated ESCs to truly participate in embryo development because of competition with host cells, or from both.

## 4. Conclusion and the future

### *4.1. Progress toward understanding pluripotency using systems biology; biomedical applications will emerge*

Progress in stem cell technology shows considerable promise for the future of biomedical research. ESCs and iPSCs can be considered the two sides of a single coin, where both have advantages and disadvantages. One of the most important potential applications of these cells is repair of diverse damaged tissues, and particularly those with limited ability for spontaneous repair. Not too long ago, organ transplantation was the only solution for this problem, and this is a technique that faces numerous obstacles, including shortage of compatible donors. ESCs and iPSCs might offer a solution to this problem as these cells have, given appropriate conditions, the potential to generate any type of somatic cell or tissue. Moreover, because iPSCs can be patient-derived to prevent any risk of transplantation rejection, this would also open the door to the generation of pluripotent stem cells without sacrificing embryos. However, ESCs might be necessary in cases, such as a heart attack or a stroke, when there is no time to generate patient-specific iPSCs, which is time-consuming, and where an off-the-shelf product is required. iPSCs have the ability to proliferate indefinitely in vitro, creating an unlimited source of cells, and the early results of iPSC differentiation studies are promising. For example, human fibroblasts have been successfully turned into iPSCs that were then differentiated into insulin-producing cells for the treatment of diabetes [85]. Mouse and human ESCs and iPSCs have been differentiated into cardiovascular cells with high efficiency [86] and, recently, the ability of human iPSCs and ESCs to differentiate into neural cells was compared, and it transpired that both cell types follow the same steps and time-course during differentiation [87]. In the rabbit, Honda et al. [26] have differentiated ESCs toward neural cells, while myocardial-like cells have also been generated from rabbit ESCs by withdrawal of LIF [88].

Rabbit ESCs and iPSCs might become the basis of important models for human disease processes. To date, several lines of putative ESCs and iPSCs have been described in the rabbit. All of these lines express stem cell-associated markers and exhibit self-renewal and pluripotency in vitro, but future validation of rabbit pluripotent stem cells would benefit greatly from the validation of a reliable panel of molecular markers specific to the characteristics of pluripotent cells in the developing rabbit embryo. Indeed, the identification and characterization of the putative pluripotency genes in the rabbit is progressing. Using rabbit-specific pluripotency genes might help to more precisely characterize rESCs and riPSCs and aid in the development of methods to more efficiently reprogram somatic cells to generate iPSCs. Identification and sequencing of rabbit-specific pluripotency genes will also aid the production of recombinant proteins and generation of specific antibodies to further characterize expression of pluripotency factors at the protein level. The ultimate goal is to resolve the difficulties in this species and produce well characterized rabbit pluripotent stem cell lines and germ line chimeras with similar efficiency to the mouse, so that they can truly be used for the envisaged biomedical applications. Moreover, the understanding of the rabbit pluripotency regulation might provide further information to help overcome similar difficulties in other species, including the pig, cow, small ruminants, and many endangered species.

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