Molecular and functional characterization of rabbit embryonic stem cell lines

Pierre Osteil, Suzy S. Markossian, Murielle Godet, Thierry Joly, Pierre Savatier, Marielle Afanassieff

SUPPORTERS:

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4th International Rabbit Biotechnology Meeting

30th June – 1st July 2011

Hungarian Academy of Sciences, Budapest

PROGRAM

30th June 2011		
Opening remarks		
9:30	**Dudits Dénes**	Vice President of Hungarian Academy of Sciences
Session 1: Rabbit genom, selection, conservation Chairman: Peter Chrenek, Jose Vicente		
10:00	**Veronique Duranthon**	Rabbit embryo as a model for genome reprogramming over preimplantation development
10:30	**Peter Chrenek**	Quality of rabbit vitrified/thawed transgenic embryos
11:00	**Coffee break, posters**	
11:30	**Csaba Pribenszky**	High pressure treatment in rabbit semen preservation
12:00	**Emmanuelle Koch**	Fetal programming analysis in the rabbit model
	Lunch	Pálinka Bistrot
Session 2: Rabbit models to study human diseases Chairman: Michael Brunner, Kazuhito Jamaguchi		
14:00	**Anne Navarette-Santos**	Rabbit as a model of embryonal development in type I diabetes women
14:30	**Koike Tomonari**	Influence of human apoAII gene on lipoprotein metabolism and atherosclerosis in transgenic rabbits
15:00	**Katja Odening**	The transgenic rabbit as a model to study the mechanisms and treatment of inborn arrhythmias
15:30	**Kazutoshi Nishijima**	Assessment of energy expenditure in rabbit with doubly-labeled water method
18:00	**Danube Corso-Budapest sightseeing cruise**	Departure from Budapest, Vigadó tér, Landing Stage, Pier 5 or 5/A

1th July 2011		
Session 3: Novel findings in rabbit ES and iPS cell establisment Chairman: Pierre Savatier, András Dinnyés		
9:00	**Arata Honda**	Generation of Induced Pluripotent Stem Cells in Rabbits: Potential experimental models for human regenerative medicine
9:30	**Pierre Savatier**	Naive and primed pluripotent stem cells in the rabbit
10:00	**András Dinnyés**	Progress and bottlenecks towards generating germline chimera forming induced pluripotent stem cells in rabbit
10:30	**Coffee break, posters**	
11:30	**Elen Gócza**	Pluripotency markers in early rabbit development and embryonic stem cells
12:00	**Pouneh Maragechi**	Stem cell specific miRNA expression in rabbit embryos and embrionic stem cells
	Lunch	Pálinka Bistrot
Session 4: Second generation methods in rabbit transgenesis Chairman: Valeri Zakhartchenko, Zsuzsanna Bősze		
14:00	**Rainer Ebel**	The zink finger nuclease technology and its perspectives in rabbit transgenesis
14:30	**Valeri Zakhartchenko**	Pluripotent and multipotent stem cells for cell-mediated transgenesis in rabbits: Chimeric and nuclear transfer animals
15:00	**Zsuzsanna Bősze**	The IgG binding Fc receptor transgenic rabbits created through BAC transgenesis
15:30	**László Hiripi**	Sleeping Beauty mediated transgenesis in rabbit
16:00	**Zsuzsanna Polgár**	Nuclear transfer technology in rabbits

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF INDUCED PLURIPOTENT STEM CELLS IN THE RABBIT

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In order to develop the induced Pluripotent Stem Cell (iPSC) technology in rabbits, we generated four rabbit iPSC lines, making use of MoMuLV-based retroviral vector that express human Oct4, Sox2, Klf4 and c-Myc to reprogram ear adult fibroblasts. The overall efficiency of iPSC derivation was estimated to 5.10^{-4}%. All four lines express the cardinal markers of pluripotent stem cells: (i) they are positive for alkaline phosphatase activity; (ii) they express the pluripotency-associated Oct4 and Nanog transcription factors, as well as the SSEA-1, SSEA-4, Tra1-60 and E-Cadherin cell surface markers; (iii) they display a normal karyotype (44XX), and (iv) they can form teratomas containing tissues of ectodermal, mesodermal and endodermal origin upon injection under the kidney capsule in SCID mice. After 25 passages, expression of all four transgenes was fully repressed in three lines out of the four analyzed, indicating complete reprogramming of fibroblasts into iPSCs. Self-renewal of rabbit iPSCs is dependent on FGF2 signaling. Upon infection with EOS – a lentiviral vector expressing the Green Fluorescent Protein (GFP) under the control of the ICM-specific distal enhancer of the mouse Pou5f1 (Oct4) gene – rabbit iPSCs show extensive fluorescence. Moreover, rabbit iPSCs display cell-cycle features that are characteristics of pluripotent stem cells, including a short G1 phase, and the lack of DNA damage checkpoint in G1 phase. The capacity of rabbit iPSCs to colonize the preimplantation embryo was explored by microinjection of GFP-expressing iPSCs into 8-cell stage rabbit embryos, and subsequent culture to the blastocyst stage. Two blastocysts, out of 65 analyzed, displayed a GFP fluorescence in the ICM.
Altogether, these results indicate that rabbit iPSCs, albeit dependent on FGF2 signaling for self-renewal, display some features of rodent iPS cells including the capacity to colonize the pre-implantation embryo.

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF RABBIT EMBRYONIC STEM CELL LINES

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We have derived four rabbit Embryonic Stem Cell (ESC) lines from New Zealand GFP-transgenic or wild-type embryos. Inner cell masses (ICMs) were isolated from 25 rabbit blastocysts by immunosurgery, and plated onto growth-inactivated murine embryonic fibroblasts in a medium supplemented with 4 ng/ml FGF2. Fifty percents were able to form an outgrowth, and 16% produced a population of highly proliferating cells that could be regularly passaged. Among the four lines obtained, one expresses the Green Fluorescent Protein (GFP) and three do not. All four Rabbit ESC lines express the pluripotency markers Oct4 and Nanog. They also express both SSEA1 and E-cadherin cell surface antigens that characterize mouse ES cells, and SSEA4, Tra1-60 and N-cadherin that characterize primate ES cells and mouse EpiSCs. Noteworthy, the percentage of SSEA1-, SSEA4-, and TRA1-60-positive cells varies considerably between ESC lines. Contrary to mouse ESCs, rabbit ESCs derived from wild-type embryos do not express the GFP after infection with the EOS lentiviral vector. EOS carries the Green Fluorescent Protein (GFP) under the control of the ICM-specific distal enhancer of the mouse Pou5f1 (Oct4) gene. To eliminate the possibility that the Pou5f1 distal enhancer is not active in the rabbit, early cleavage stage rabbit embryos were infected with EOS, and subsequently cultured until the blastocyst stage. Confocal microscopy analysis revealed the presence of fluorescent cells within the ICM. Furthermore, after ICM isolation, infection with EOS, and subsequent plating, GFP-positive cells were visible in the resulting outgrowths, but fluorescence disappeared after 48 hours. Therefore, we conclude that the Pou5f1 distal enhancer is active in rabbit embryonic stem cells in vivo, but its activity is rapidly lost upon in vitro culture. All ESC lines tested display the capacity to make teratomas after injection beneath the kidney capsule in SCID mice. All teratomas contain derivatives of the three embryonic germ layers, demonstrating the pluripotent nature of these cell lines. Contrary to mouse and primate ESCs, rabbit ESCs exhibit a long G1 phase in that as much as 50% of the SSEA1-positive cell fraction displays a 2n DNA content. Surprisingly, of the four ESC lines analyzed, two exhibited a DNA damage checkpoint in G1 like somatic cells, whereas the other two, like mouse ESCs and EpiSCs, did not. We can conclude from these studies that rabbit ESC lines are heterogeneous in nature, with only some lines showing the cell-cycle cardinal features of pluripotent stem cells.
We also explored the capacity of rabbit ESCs to colonize the pre-implantation embryo. To this aim, we used the GFP-expressing ESC line that does not exhibit a DNA damage checkpoint in G1. Dually GFP/SSEA1-positive cells were FACS-sorted, subsequently micro-injected into 8-cell stage embryos, and the resulting embryos cultured to the blastocyst stage. No evidence of GFP-positive cells in the ICM was found from 58 embryos analysed.
Altogether, these results indicate that rabbit ESCs do not all exhibit the cell-cycle cardinal features of pluripotent stem cells. Moreover, they are unable to participate in embryo development in vivo.

MOLECULAR AND FUNCTIONAL CHARACTERIZATIONOF RABBIT EMBRYONIC STEM CELL LINES

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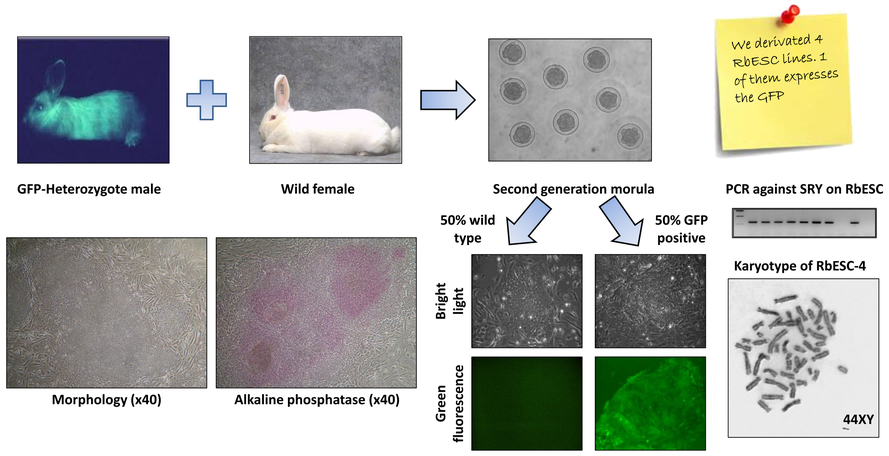
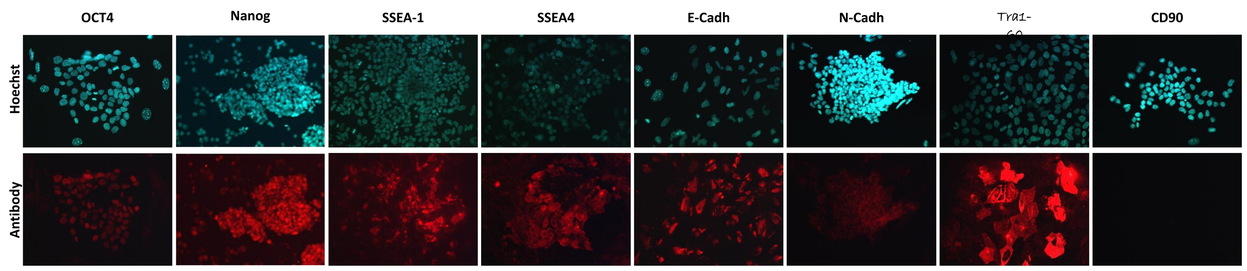
Introduction

We have derived four Rabbit Embryonic Stem Cell (RbESC) lines from New Zealand GFP-transgenic and wild-type embryos. Inner cell masses (ICMs) were isolated from 25 rabbit blastocysts by immunosurgery, and plated onto growth-inactivated murine embryonic fibroblasts in a medium supplemented with 13ng/ml FGF2. Fifty percents were able to form an outgrowth, and 16% produced a population of highly proliferating cells that could be regularly passaged.

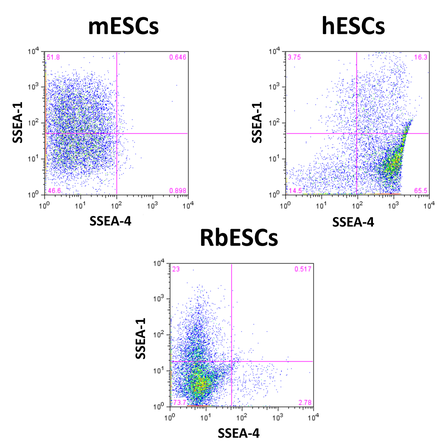
1. Establishment of rabbit embryonic stem cell (RbESC) lines



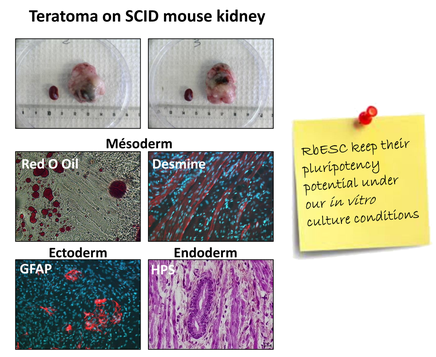
2. Pluripotency markers

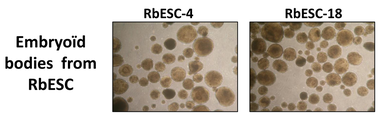


Lines	mESc	RbFc	Rh ESc	RbES-4		RbES-8		RbES- 18	RbES-19	
Day of culture	1	3	3	1	3	1	3	3	1	3
OCT4	+	-	++	+	+	+	+	+	+	+
Nanog	+	-	++	+	+	+	+	+	+	+
SSEA1	+	-	-	+	-	+	-	+	+	+
SSEA4	-	-	++	-	-	-	-	-	-	+
CD90	-	-	++	-	-	-	-	-	-	-
TRA-1-60	-	-	++	-	-	-	+	-	-	-
N-Cadh	-	-	+	+	+	+	+	+	+	+
E-Cadh	+	-	+	+	+	+	+	+	+	+

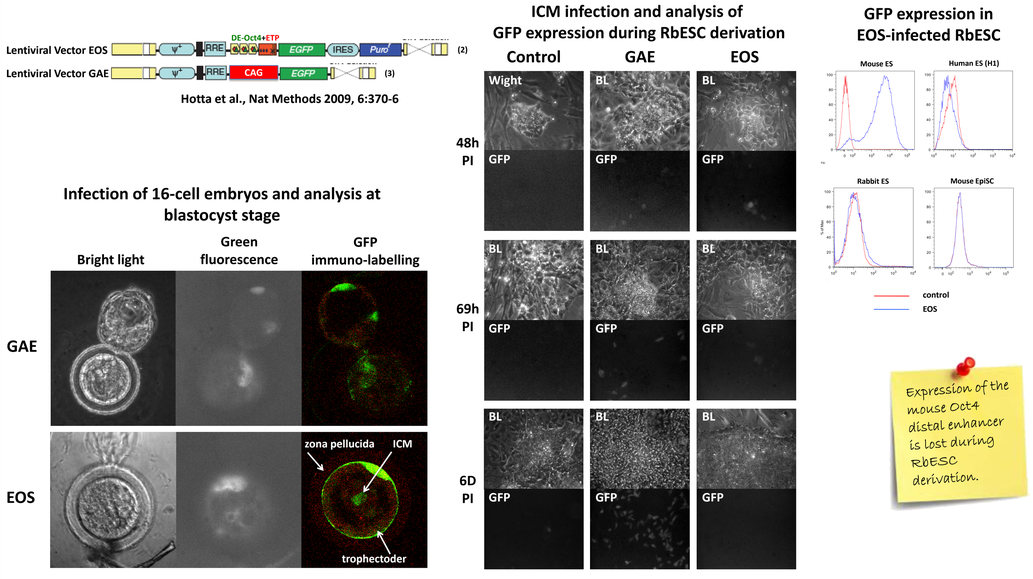


3. Pluripotency

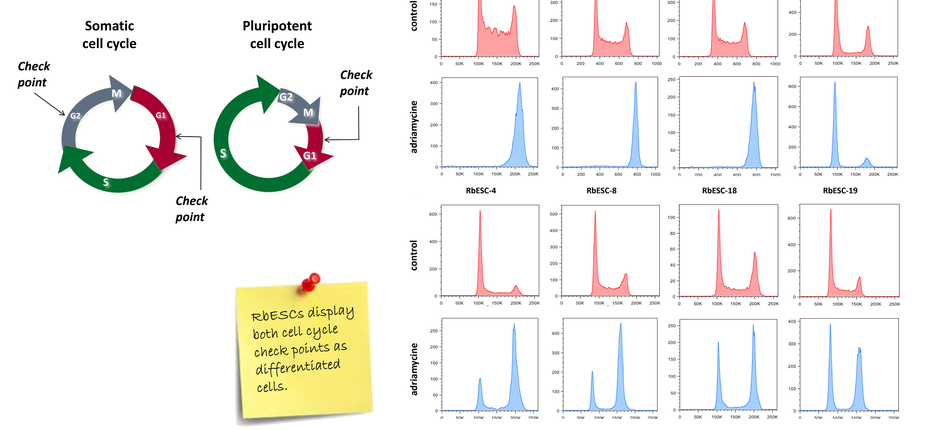




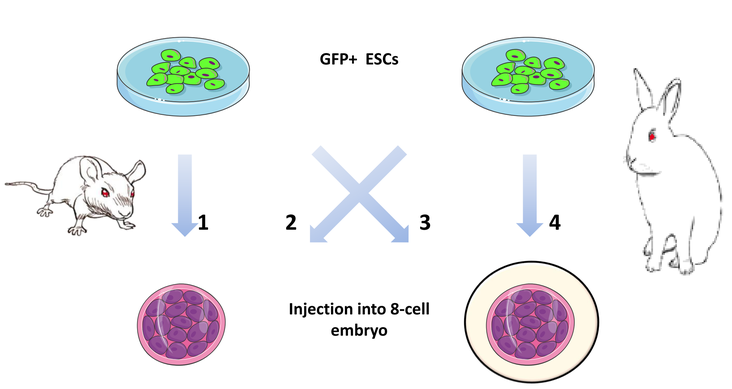
4. Expression of the distal Enhancer of mouse Oct4 gene in RbESCs

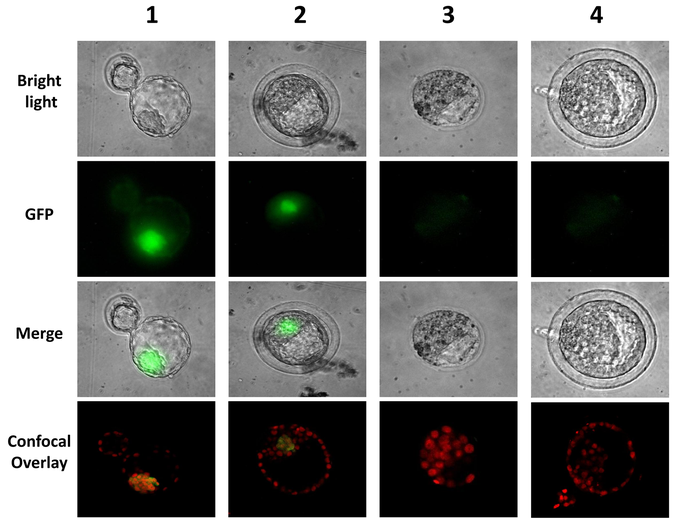


5. Cell cycle of RbESCs



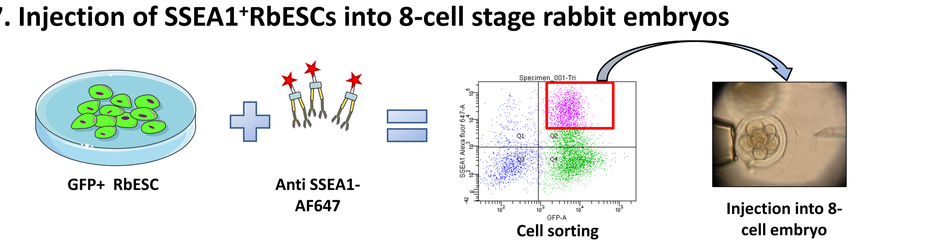
6. Interspecies injection of mESCs and RbESCs into 8-cell stage embryos

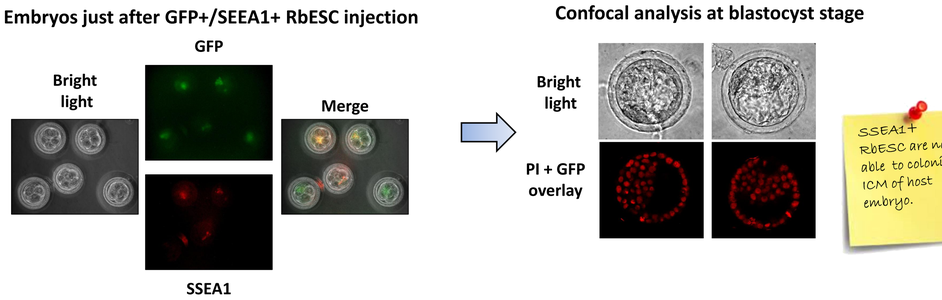




RbESCs fail to colonize mouse and rabbit pre-implantation embryos

7. Injection of SSEA1⁺RbESCs into 8-cell stage rabbit embryos





Conclusion

Our results indicate that rabbit ESCs exhibit a heterogeneous profile of pluripotency markers and do not all show the cell-cycle cardinal features of pluripotent stem cells. Furthermore, they are unable to participate in embryo development *in vivo*.