



Molecular and functional characterization of induced pluripotent stem cells in the rabbit

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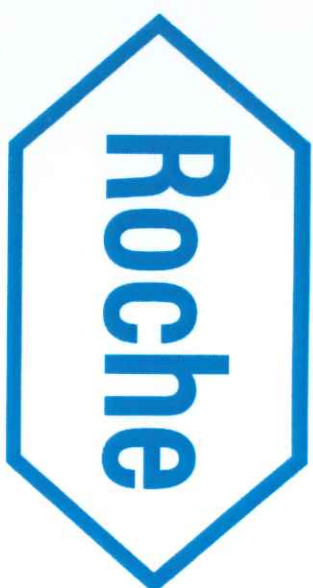
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**4th International Rabbit
Biotechnology Meeting**

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 Duranton	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		
Session 2: Rabbit models to study human diseases		
Chairman: Michael Brunner, Kazuhito Jamauchi		
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 establishment		
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 Góczy	Pluripotency markers in early rabbit development and embryonic stem cells
12:00	Pounch Maragechi	Stem cell specific miRNA expression in rabbit embryos and embryonic stem cells
Lunch		
Session 4: Second generation methods in rabbit transgenesis		
Chairman: Valeri Zakharichenko, Zsuzsanna Bősze		
14:00	Rainer Ebel	The zinc finger nuclease technology and its perspectives in rabbit transgenesis
14:30	Valeri Zakharichenko	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 5.10⁻⁶%. All four lines express the cardinal markers of pluripotent stem cells: (i) they are positive for alkaline phosphatase activity; (ii) they express 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*.

Rabbit iPSCs display some features of murine iPSCs, including SSEA1 expression, Oct4 distal enhancer activity and limited capacity to colonize the pre-implantation embryo.