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Pierre Osteil, Yann Tapponnier, Anaïs Moulin, Thierry Joly, Pierre Savatier, Marielle Afanassieff

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THE RABBIT PRE IMPLANTATION EMBRYO AS A PARADIGM TO EXPLORE NAIVE EMBRYONIC STEM CELL DERIVATION IN NON RODENT SPECIES

Author Block: OSTEIL, Pierre[1], TAPPONNIER, Yann[1], MOULIN, Anaïs[1], JOLY, Thierry[2], SAVATIER, Pierre[1], AFANASSIEFF, Marielle[1]

[1]Stem cell and Brain Research Institute, INSERM U846, INRA USC1361, BRON, France, [2]VetAgroSup, UPSP ICE, ISARA-Lyon, Lyon, France

Abstract:
The scarcity of pre-implantation embryos in human is a major limitation to the improvement of embryonic stem cell (ESC) derivation in non-rodent species. Rabbit is a good alternative as it can produce up to 30 embryos per super-ovulated females. Moreover, all rabbit ESC line produced so far showed the characteristics of primed pluripotency like human ESCs. In this work, we investigated the conditions suitable for the generation of naïve ESCs in rabbits. A first experiment aimed to study the effect of the pharmacological inhibitor of MEK signaling, PD0325901, on embryo development and ESC line derivation. We collected 576 eight-cell stage embryos, of which 317 were cultured in the presence of MEK inhibitor until they reached the early blastocyst stage. No difference in the rate and quality of embryo development between MEK inhibitor-treated and control embryos was observed. ICMs were isolated by immunosurgery and plated onto gelatin- or fibronectin-coated dishes in various media (DMEM/F12 + 20% KOSR, N2B27) supplemented with GSK3 inhibitor (CHIR99021), MEK inhibitor, and LIF (2i/LIF), or not. 80% of the ICMs plated, but none could be expanded beyond passage 2. We concluded that inhibition of MEK signaling fails to prevent spontaneous differentiation of pluripotent stem cells in rabbit. A second experiment aimed to study the effect of LIF on ESC line derivation. We collected 262 ICMs, which were plated onto growth-inactivated mouse embryonic fibroblasts in DMEM/F12 supplemented with 20% KOSR (72), 20% KOSR + LIF (90), or 10% KOSR + 10% FCS + LIF (80). No outgrowth cultured in media lacking LIF could be expanded beyond passage 4. By contrast, 7 ESC lines were derived from outgrowths cultured and expanded in the presence of LIF, 2 in 20% KOSR + LIF, and 5 in 10% KOSR + 10% FCS + LIF. Two lines, designated rbES-LIF1 and rbES-LIF2, were expanded by gentle dissociation with collagenase until passage 40, and showed a normal karyotype. RbES-LIF1 and rbES-LIF2 displayed the cardinal features of pluripotent stem cells, i.e. expression of pluripotency markers, differentiation into derivatives of the 3 germ layers, and teratoma formation. However, they could not be cultured onto gelatin-coated dishes, they did not express markers associated with naïve pluripotency in rodents, and they did not survive in 2i/LIF medium. We concluded that LIF facilitates the derivation of ESCs but does not support naïve pluripotency in rabbits. When rbES-LIF1 and rbES-LIF2 cells were propagated for 20 passages in 10% KOSR + 10% FCS + LIF, and were enzymatically dissociated with Accutase into single cell suspensions, they acquired

chromosomal abnormalities (43XX; 45XY). The same observation was made with the 5 freshly derived ESC lines in 10% KOSR + 10% FCS + LIF with Accutase, of which 3 displayed abnormal chromosome numbers. We concluded that LIF-dependent rabbit ESCs cannot be propagated under stringent conditions without frequent chromosomal rearrangement-based adaptation.

Travel Grant Justification:

In order to be able to attend to the ISSCR meeting in Boston and to present my PhD work, I will be pleased to obtain a travel grant of 900€ (about 1200$) corresponding to the price of plane tickets from Lyon (France) to Boston.

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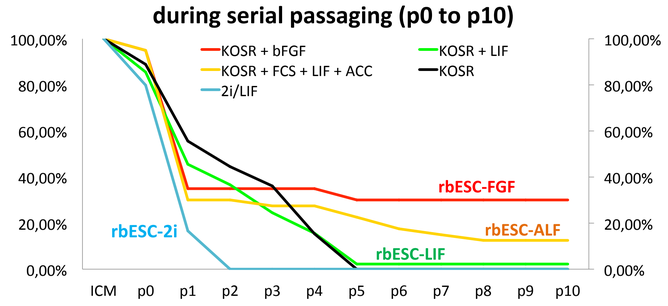
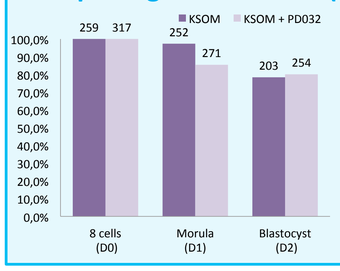
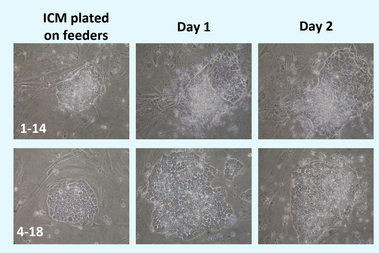
INTRODUCTION: the scarcity of pre-implantation embryos in human is a major limitation to the improvement of embryonic stem cell (ESC) derivation in non-rodent species. Rabbit is a good alternative as it can produce up to 30 embryos per super-ovulated females. Moreover, all rabbit ESC line produced so far showed the characteristics of primed pluripotency like human ESCs. In this work, we investigated the conditions suitable for the generation of naïve ESCs in rabbits.

Percentage of expanding ICM outgrowths during serial passaging (p0 to p10)



I- Pre-implantation embryo development, and ICM growth after plating onto feeders (+/- MEK inhibition with PD032)



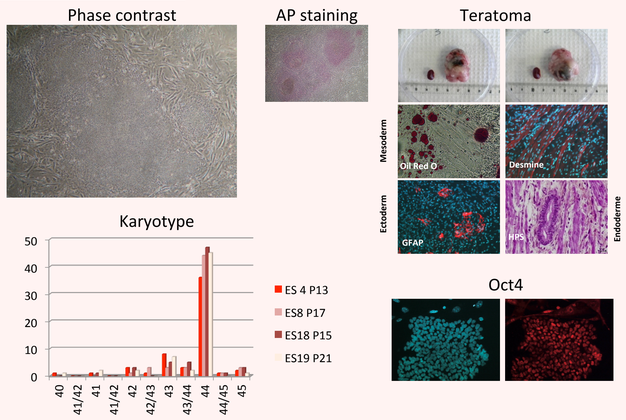


No difference between PD032-treated and untreated embryo in the rate of successful development was observed. ICM cultured in PD032 failed to generate ESC lines.

II-RbESC-FGF

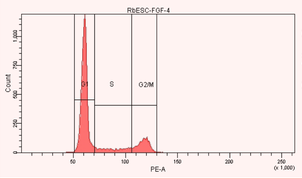
Serum : 20% KOSR — Feeders: MEF (from OF1 mice)
Growth factor : FGF2 — Passaging: Clumps (Collagenase)

A) Characterization



RbESC-FGF cells display the cardinal features of embryonic stem cells. They express pluripotency markers, they form teratomas, and they have a stable karyotype (44XY).
Details are available in *Osteil, Tapponnier, Markossian et al.* Induced pluripotent stem cells derived from rabbits exhibit some characteristics of naïve pluripotency (*2013) Biology Open*

B) Cell cycle analysis

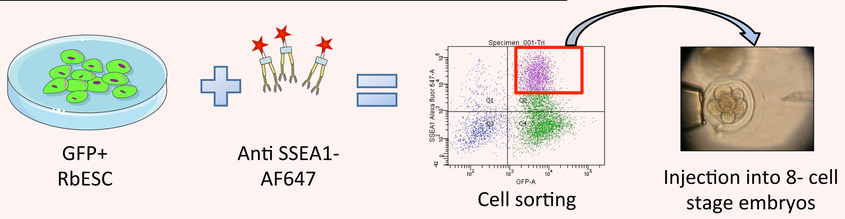


G1 : 72.7%
S : 11%
G2/M : 16.3%

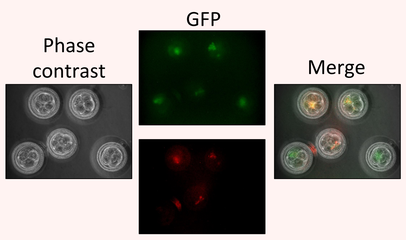
RbESC-FGF cells have a poor growthy rate and exhibit a long G1 phase.

G1 phase duration is an important feature of naïve cells. See *Coronado et al.* A short G1 phase is an intrinsic determinant of naïve embryonic stem cell pluripotency. *(2013) Stem Cell Research*

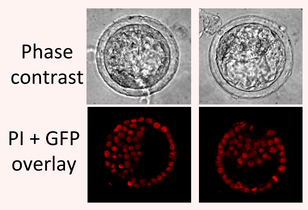
C) ICM colonization after injection into 8-cell stage embryos



Embryos at 1 hour post injection



Confocal analysis at blastocyst stage

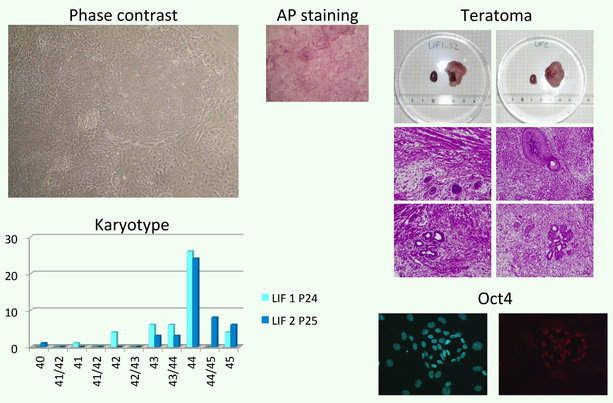


One hundred and twelve 8-cell stage embryos were injected with 10-15 cells, and further cultured to the blastocyst stage. No blastocyst with GFP positive cells in the ICM could be observed.

III-RbESC-LIF

Serum : 20% KOSR — Feeders: MEF (from OF1 mice)
Growth factor : LIF — Passaging method: Clumps (Collagenase)

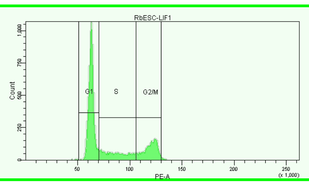
A) Characterization



RbESC-LIF cells display the cardinal features of embryonic stem cells. They express pluripotency markers, they form teratomas, and they have a stable karyotype (44XY).

B) Cell cycle

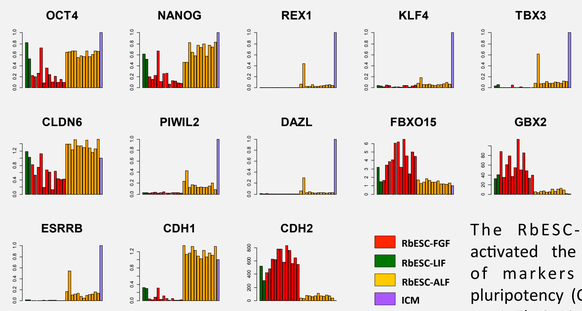
G1 : 55.9%
S : 20.2%
G2/M : 23.9%



RbESC-LIF cells exhibit a shorter G1 phase as compared to RbESC-FGF.

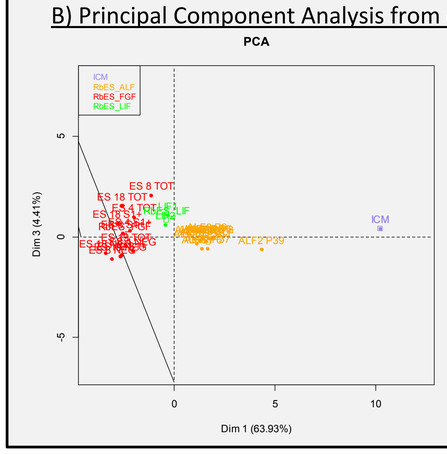
V- Gene expression analysis

A) Barplots from qPCR data



The RbESC-ALF cells activated the expression of markers of naïve pluripotency (Cdh1, Esrrb, Rex1, Tbx3, Piwil2, Dazl)

B) Principal Component Analysis from qPCR data

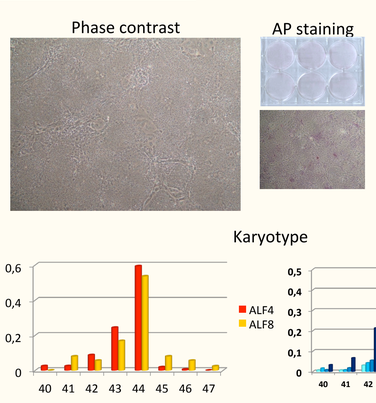


A selection of pluripotency-associated genes was analyzed by quantitative RT-PCR. Followed by a PCA analysis According to the molecular signatures, the RbESC-FGF are the furthest from the ICM, whereas the RbESC-ALF cells are the closest. RbESC-LIF cells exhibit an intermediate signature

IV-RbESC-ALF

Serum : 10% KOSR + 10%FCS — Feeders: MEF (from OF1 mice)
Growth factor : LIF — Passaging method: Single cell suspension (Accutase)

A) Characterization

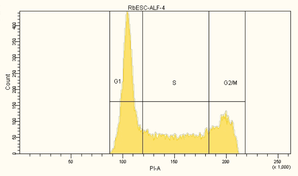


RbESC-ALF cells are pluripotent (characterization is ongoing). However, they acquired chromosomal abnormalities during passagings (43XX, 45XY)

Two RbESC-ALF lines have a relatively more stable karyotype (44XX)

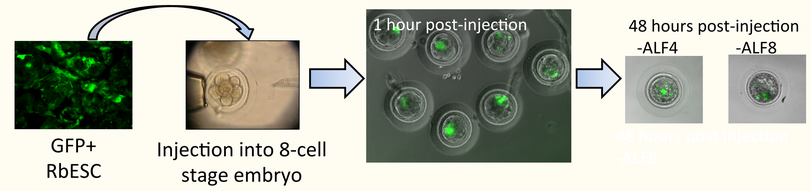
B) Cell cycle analysis

G1 : 46.6%
S : 31.9%
G2/M : 21.5%



RbESC-ALF cells have a cell-cycle distribution characterized by a smaller fraction of cells in G1 phase as compared to other rabbit ES cells.

C) ICM colonization after injection into 8-cell stage embryos



RbESC lines	ALF2	ALF4	ALF6	ALF8	ALF10	ALF12	TOTAL
Injected embryos (8-cell stage embryos)	41	68	42	57	45	50	303
Positive embryos +48h	2	12	10	7	12	5	48
Positive blastocysts +48h	0	2	4	1	2	0	9

Of 303 embryos that developed to the blastocyst stage, 9 had GFP positive cells in the ICM.

CONCLUSIONS:

1/ Inhibition of MEK signaling prevents ICM outgrowths from generating ESCs in rabbit.

2/ Rabbit ESCs derived in Fetal Calf Serum on feeders, and dissociated to single cell suspension during passaging (RbESC-ALF), exhibit an elevated expression of genes whose expression is associated with naive pluripotency in rodents.

3/ RbESC-ALF cells exhibit a shorter G1 phase.

4/ RbESC-ALF cells can colonize the ICM of the rabbit blastocyst following injection into 8-cell stage embryos

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