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Isolation of rabbit and goat ES-like cells with human ES cell features

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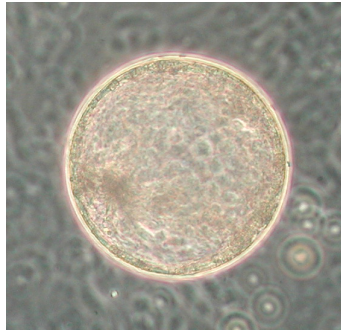


ISOLATION OF RABBIT AND GOAT ES-LIKE CELLS WITH HUMAN ES CELL FEATURES

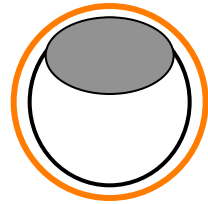
**MARKOSSIAN Suzy, SAVATIER Pierre and
AFANASSIEFF Marielle**

**Stem Cell and Brain Research Institute
PrimaStem**

Rabbit and Goat ESC derivation

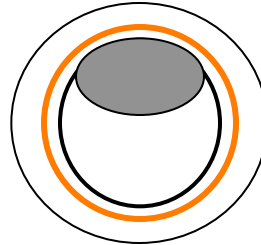


Goat Blastocyst



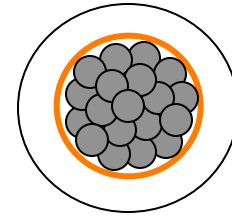
Blastocyst without Mucus coat

Pronase

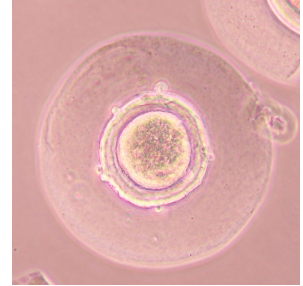


Blastocyst

Culture

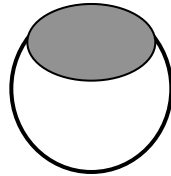


Morula

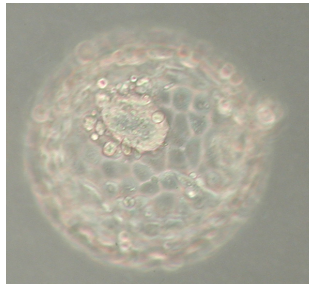


Rabbit Morula

Mécanique

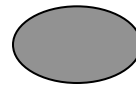


Blastocyst without zona pellucidae

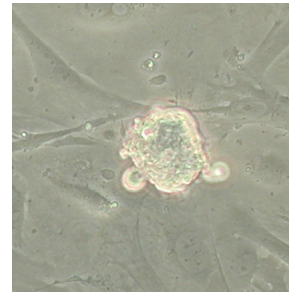


Rabbit Blastocyst Without zona pellucidae

Immunosurgery



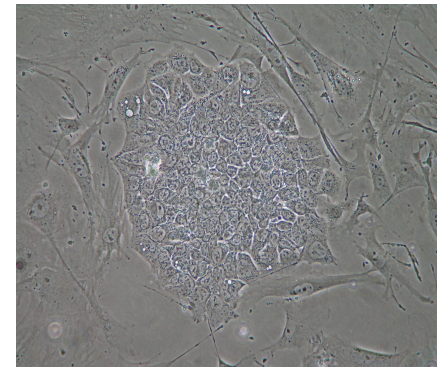
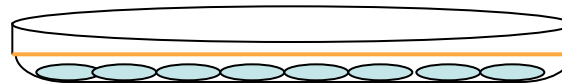
Inner Cell Mass



Rabbit ICM

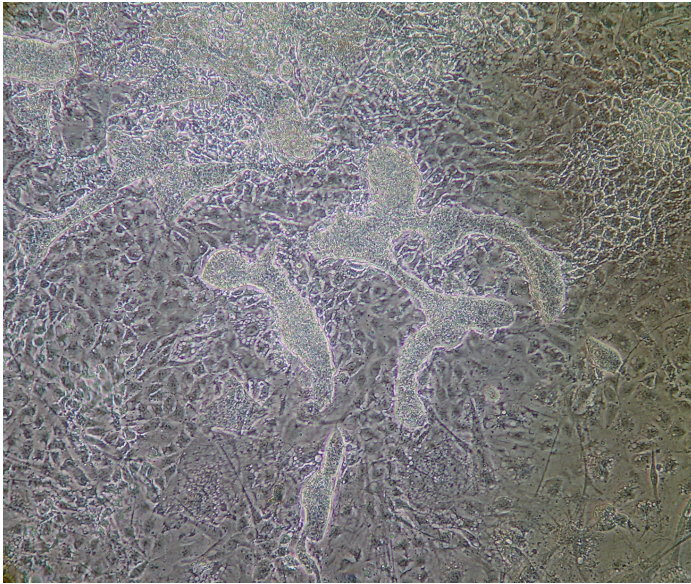
Rabbit ICM after 20h culture on MEF

Culture on MEF



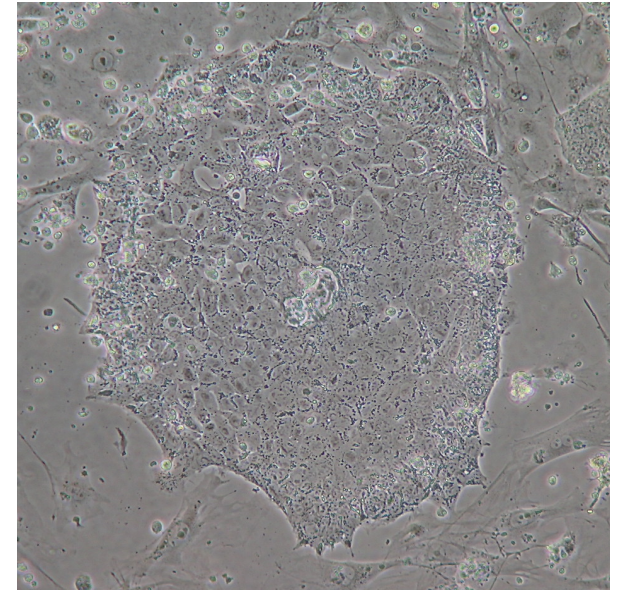
Culture Medium :
KO DMEM + 10% FBS + 10% KO SR + FGF2
(medium using to derive monkey ES line: LyonES)

Morphology of primary and secondary colonies

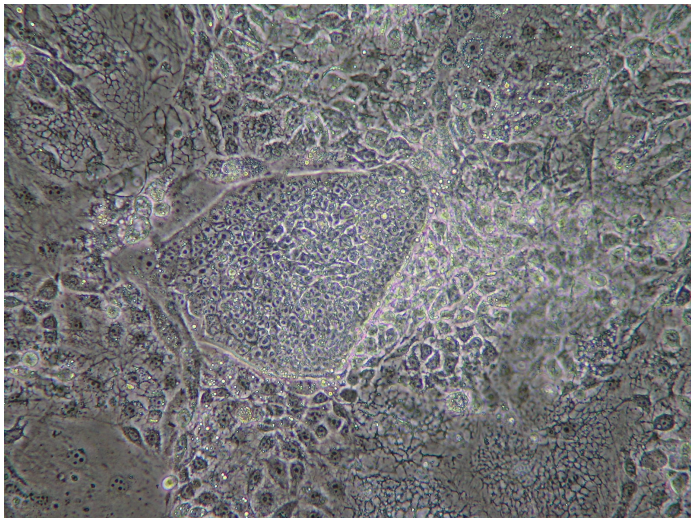


Primary culture of Rabbit ICM
Appearance of outgrowths after 5 days

→
Mecanical dissociation

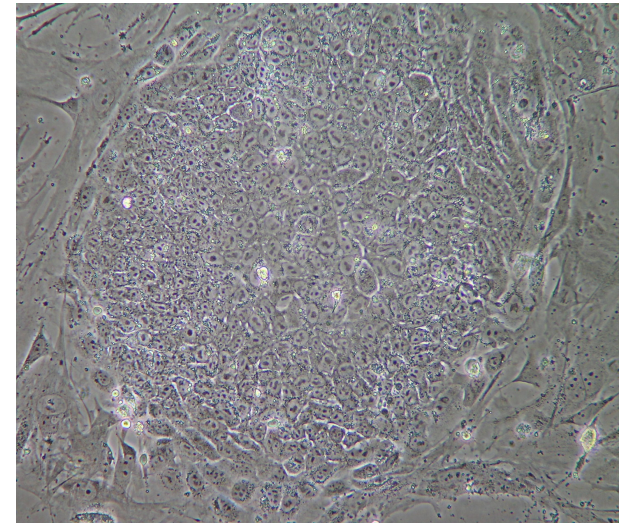


Rabbit ES-like P2 colony



Primary culture of Goat ICM
Appearance of outgrowths after 3 days

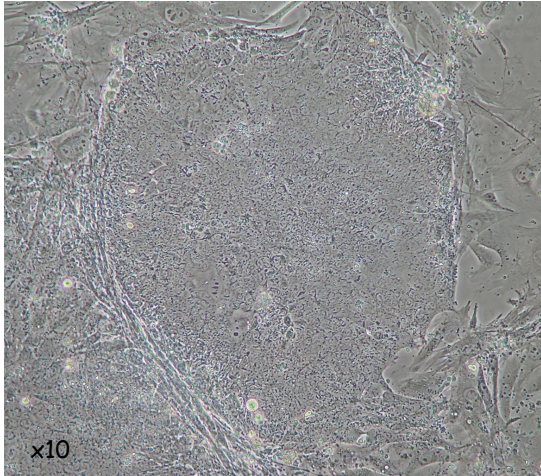
→
Collagenase and
Mechanical dissociation



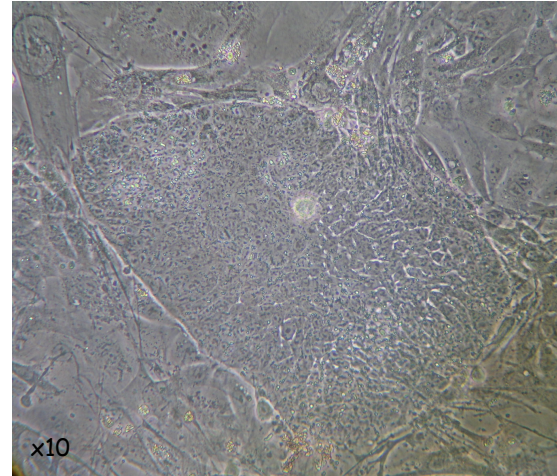
Goat ES-like P2 colony

Morphology of secondary ES-like colonies

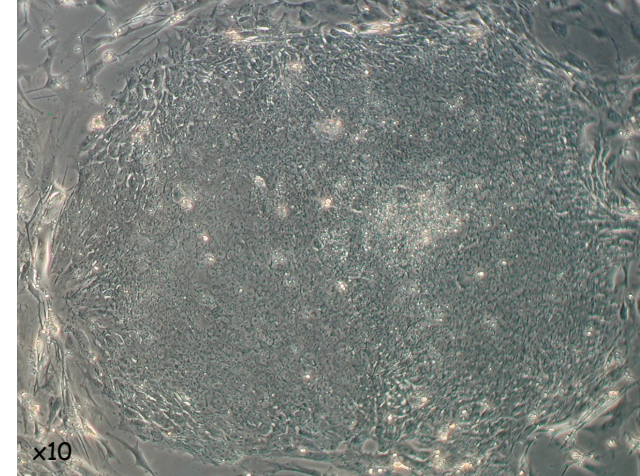
Rabbit ES-like colony



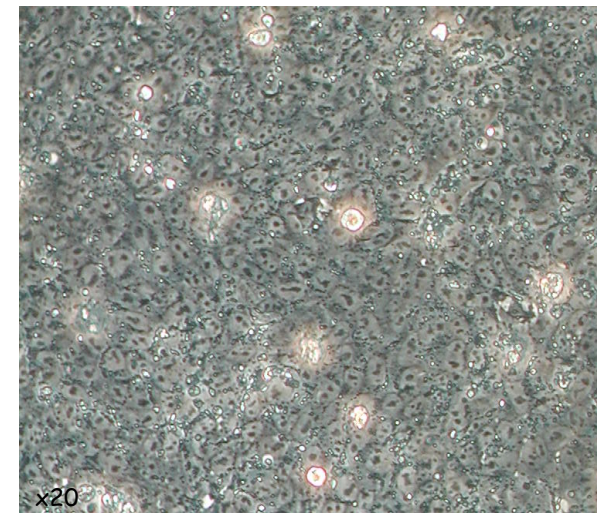
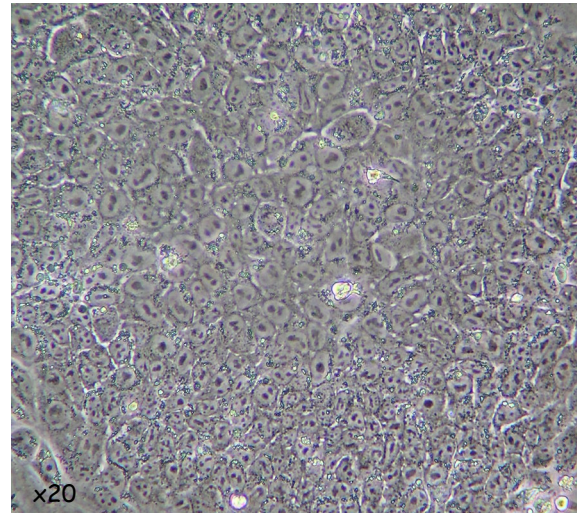
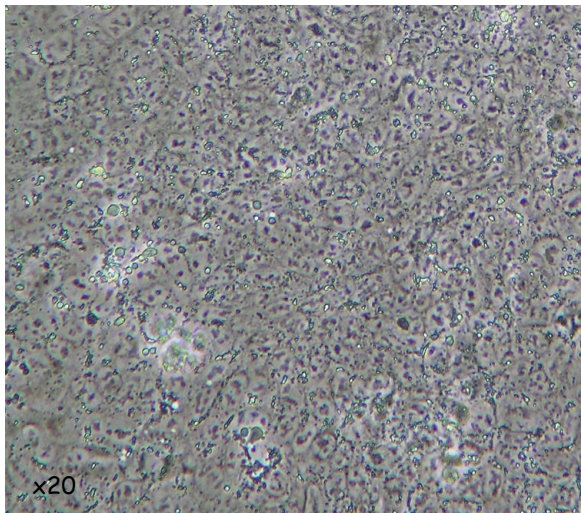
Goat ES-like colony



Human ES colony

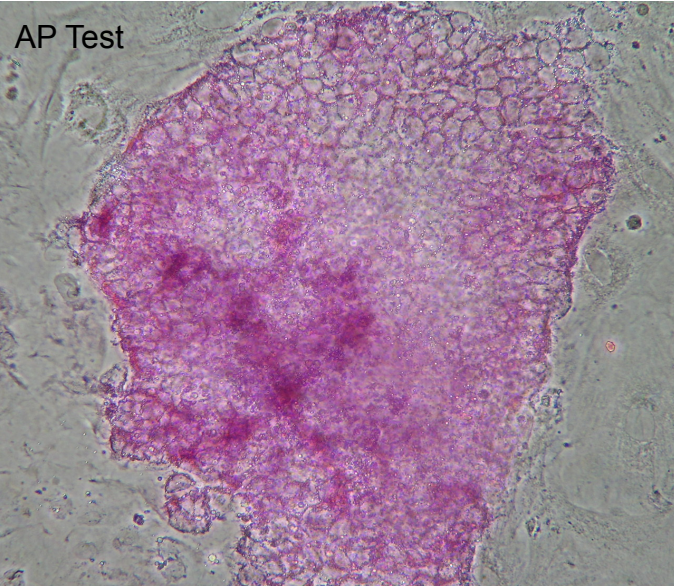
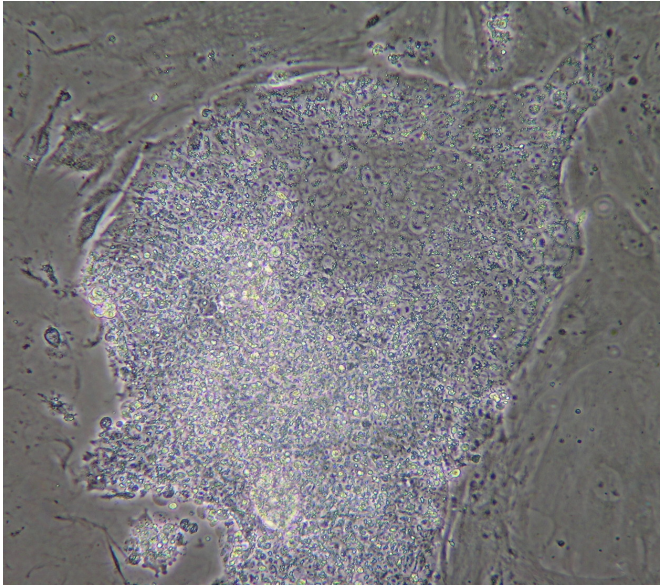


➤ Flat colonies of compact cells

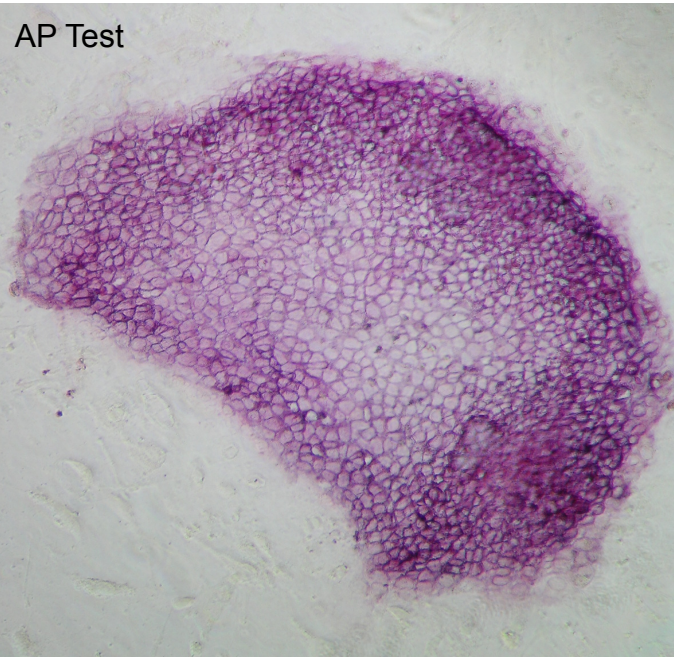
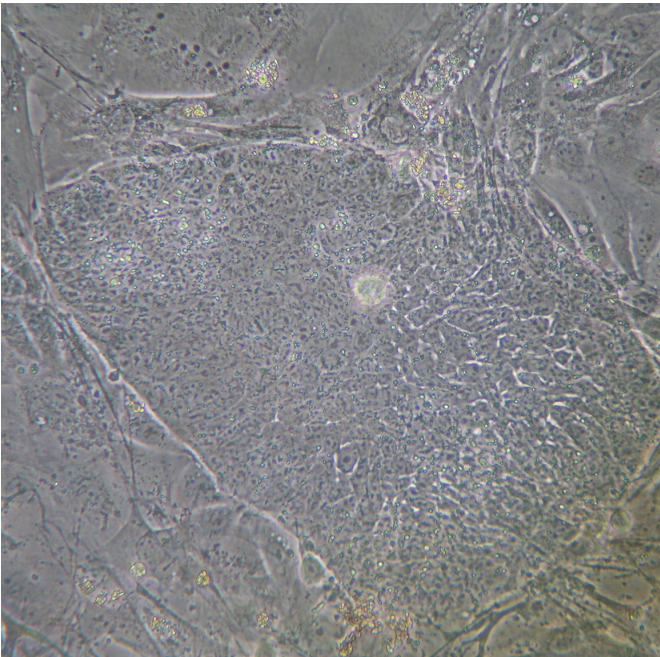


➤ High nucleus/cytoplasm ratio and prominent nucleoli

Alkaline phosphatase activity in ES-like cells



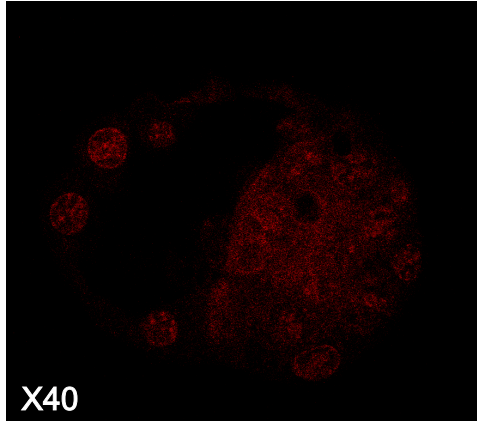
Rabbit ES-like colony



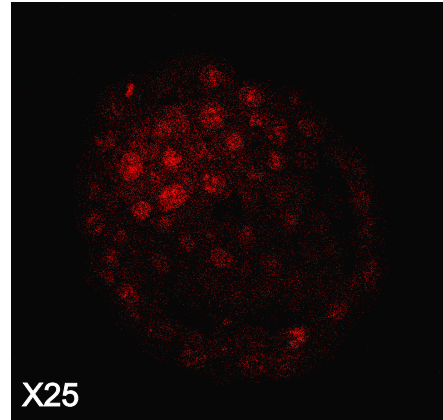
Goat ES-like colony

Oct4 expression in blastocyst cells

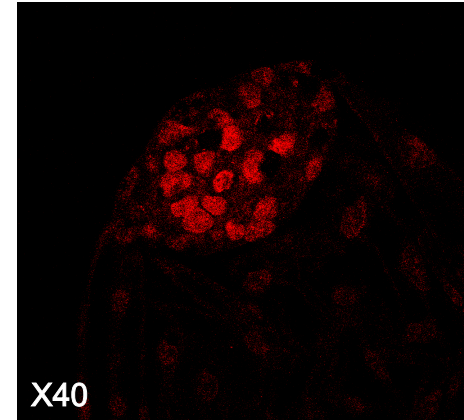
Mouse



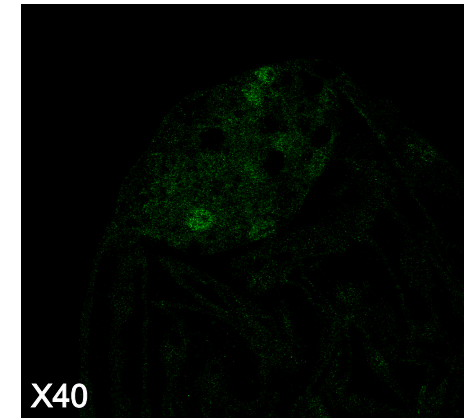
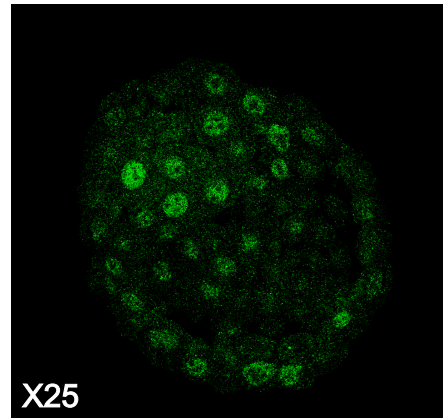
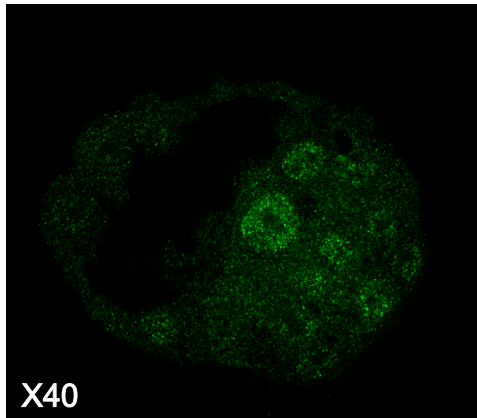
Rabbit



Goat



Nucleus
labelling
by propidium
iodide



Anti-Oct4
immuno-
fluorescence
confocal
microscopy

- High Oct4 expression in ICM cells
- Lower Oct4 expression in trophectoderm cells

Oct4 expression in rabbit ES-like cells

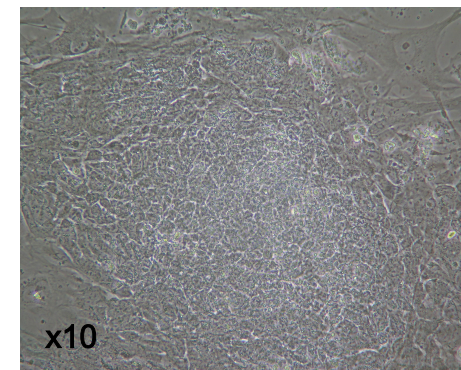
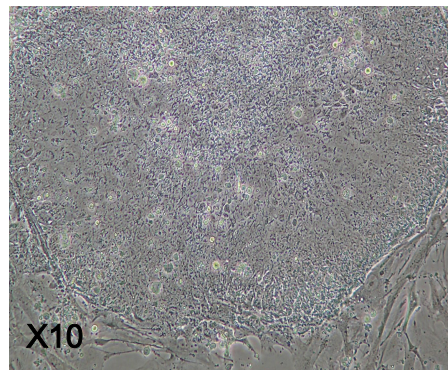
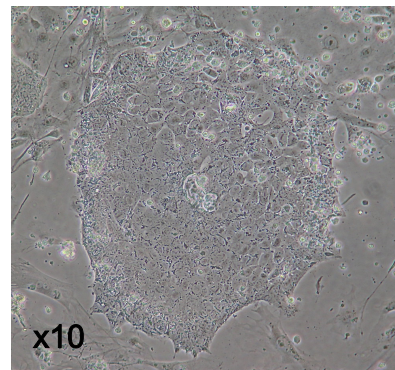
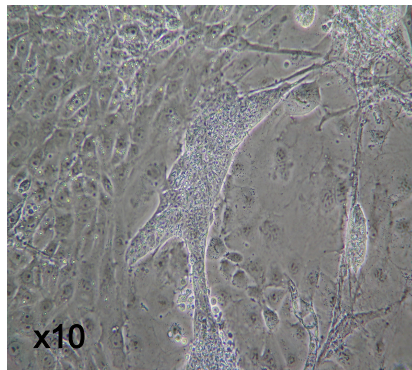
Passage 1

Passage 2

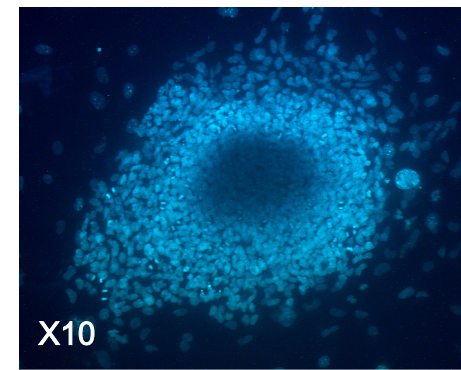
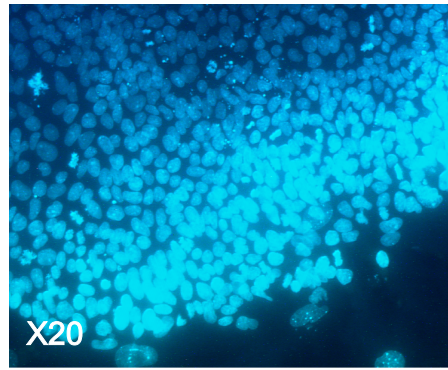
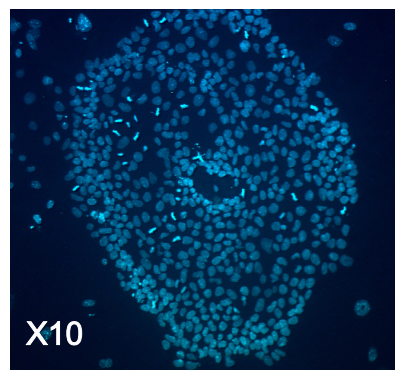
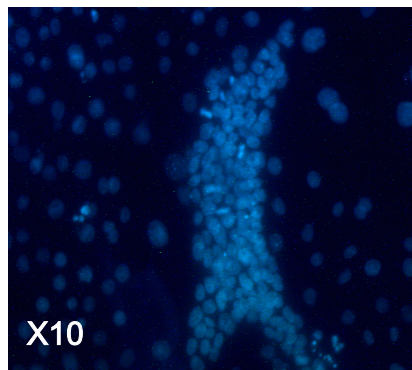
Passage 4

Passage 6

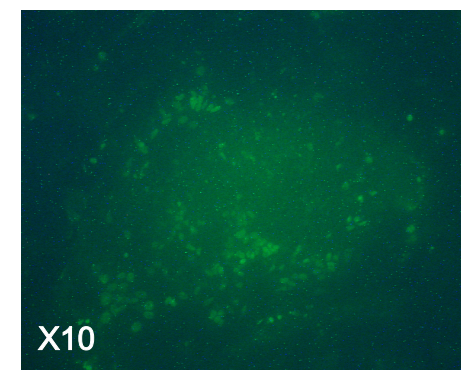
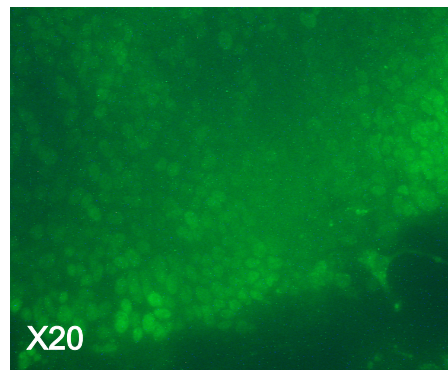
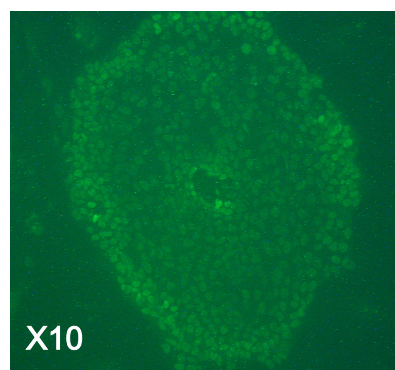
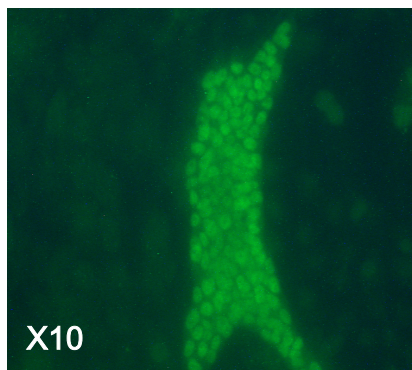
Contrast of Phase



Hoechst



Anti-Oct4



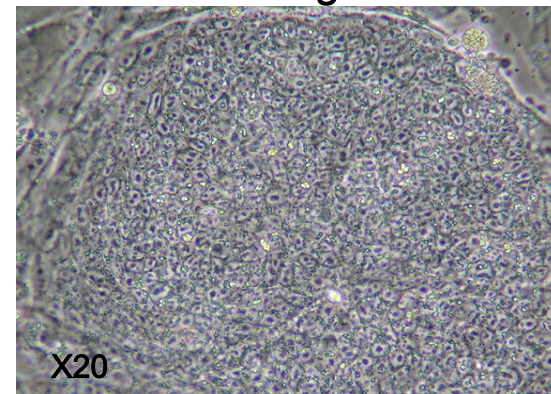
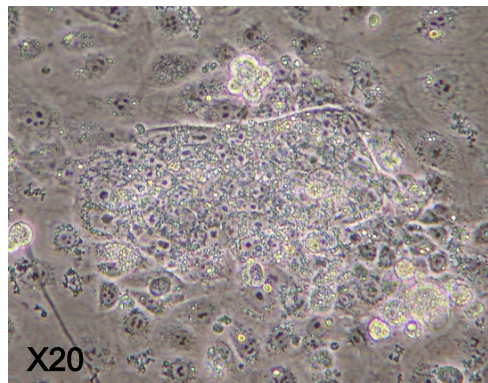
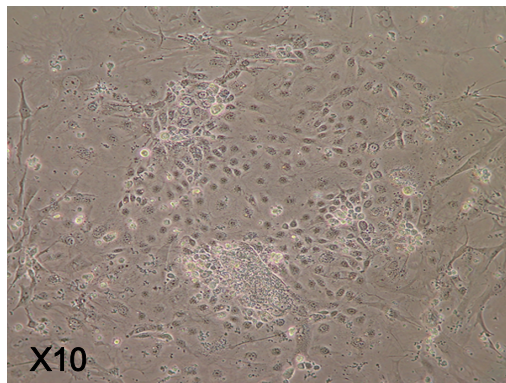
- Loss of Oct4 expression with ES-like cell differentiation
- Self-renewal of ES-like cells is not sustained in applied culture conditions

Oct4 expression in goat ES-like cells

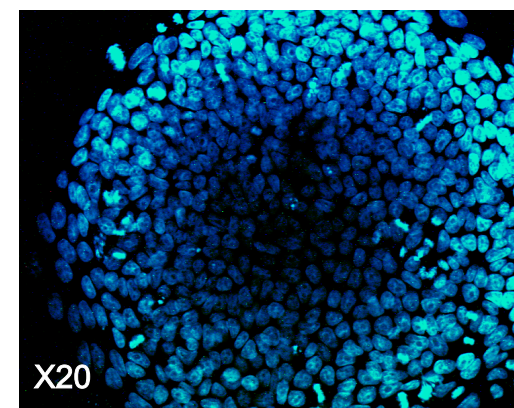
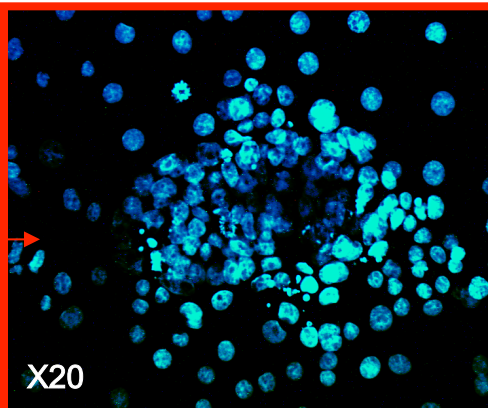
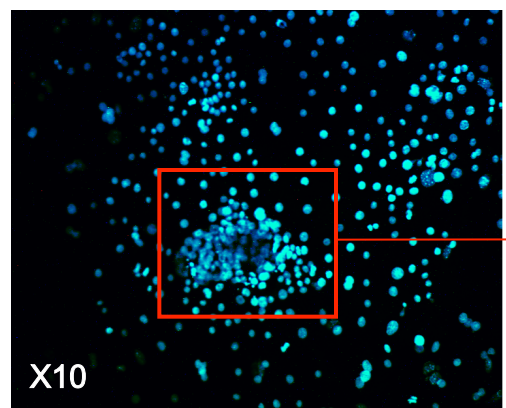
Passage 1

Passage 2

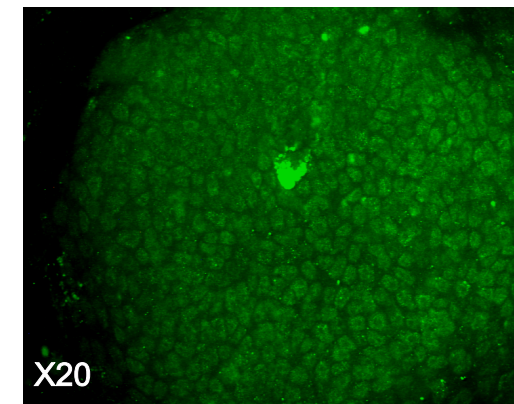
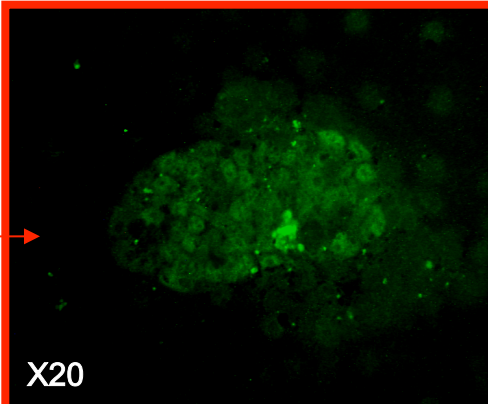
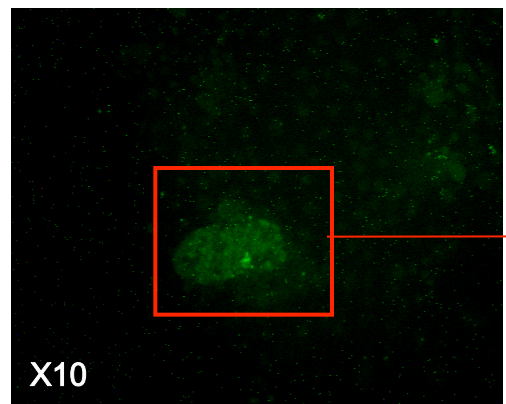
Contrast of Phase



Hoechst



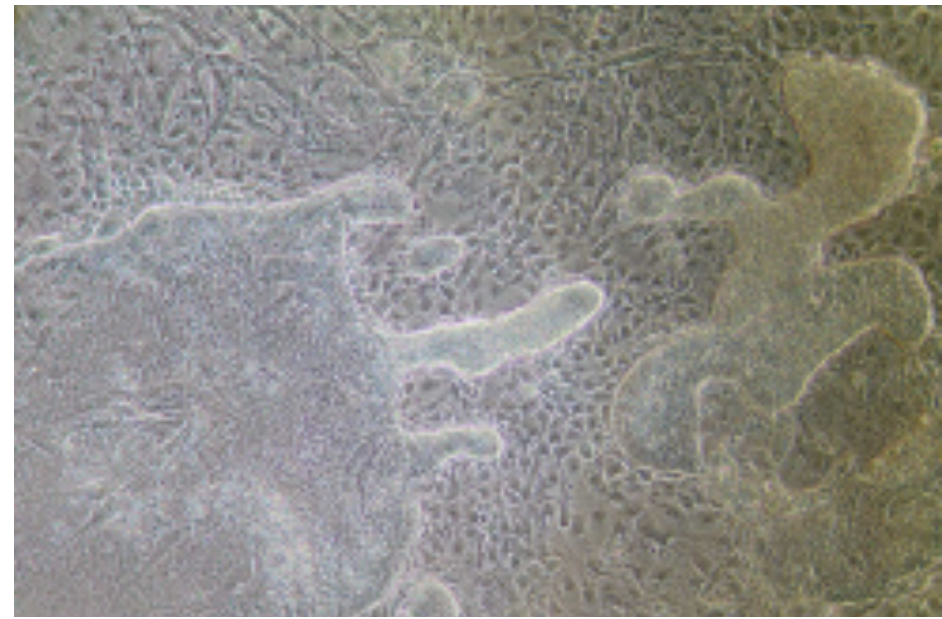
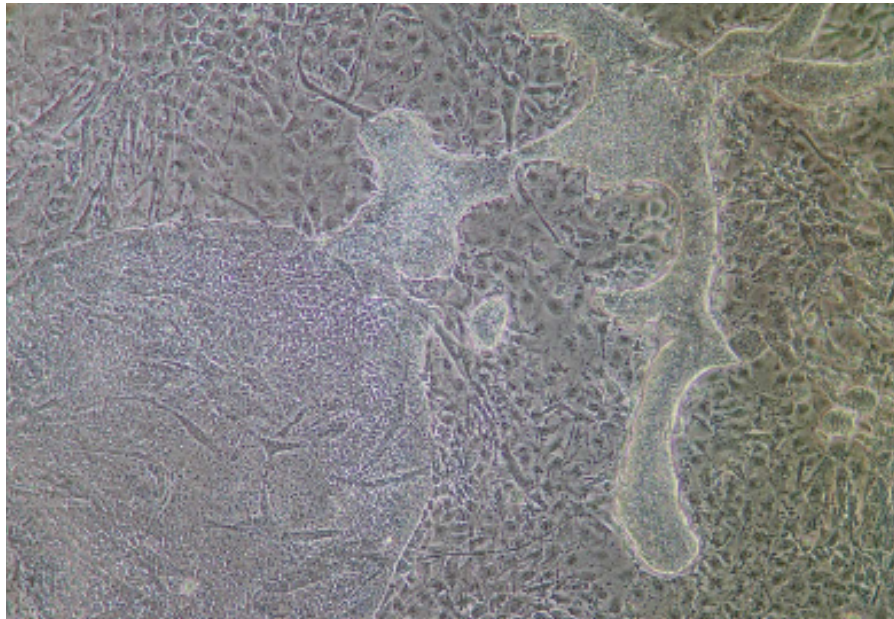
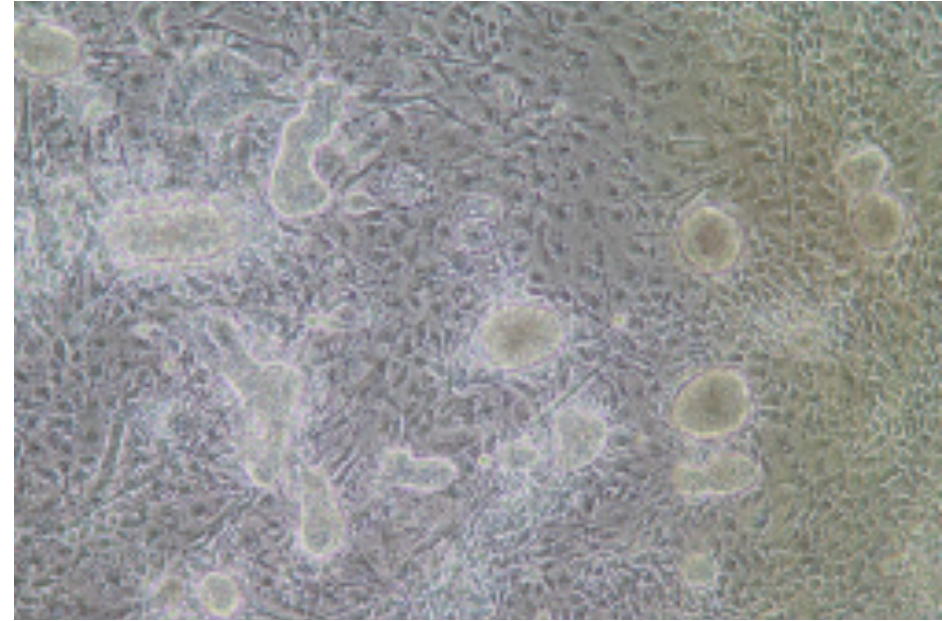
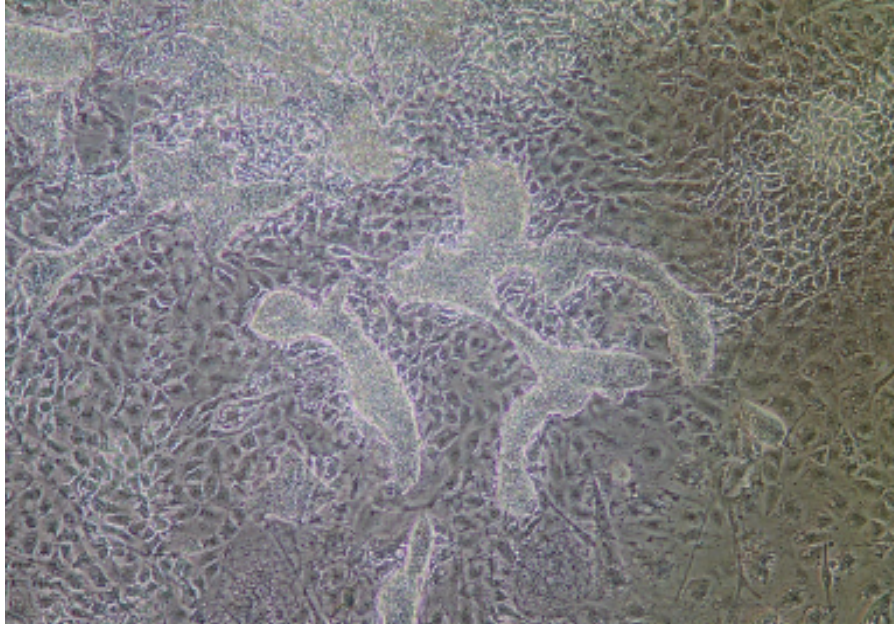
Anti-Oct4



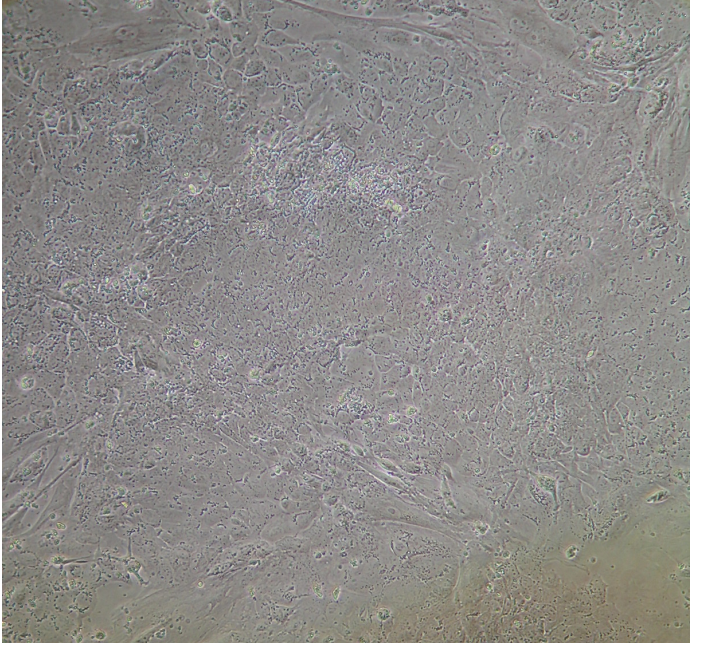
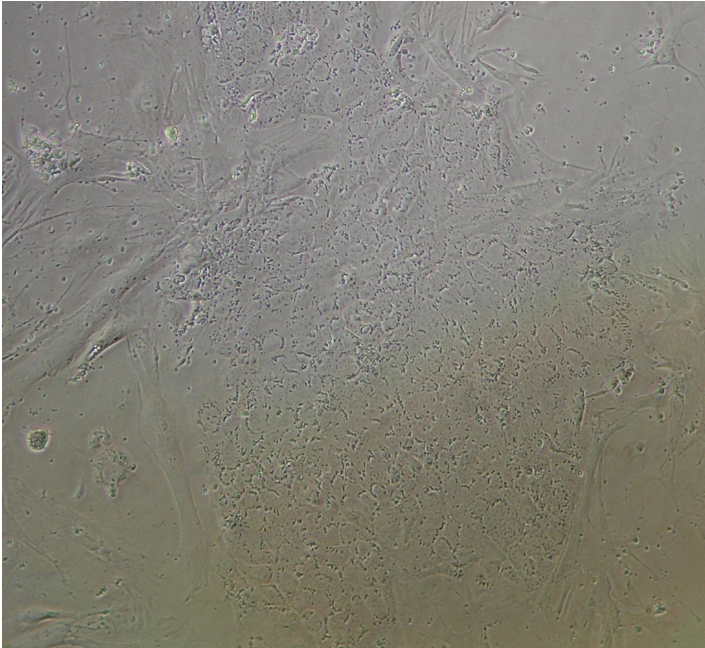
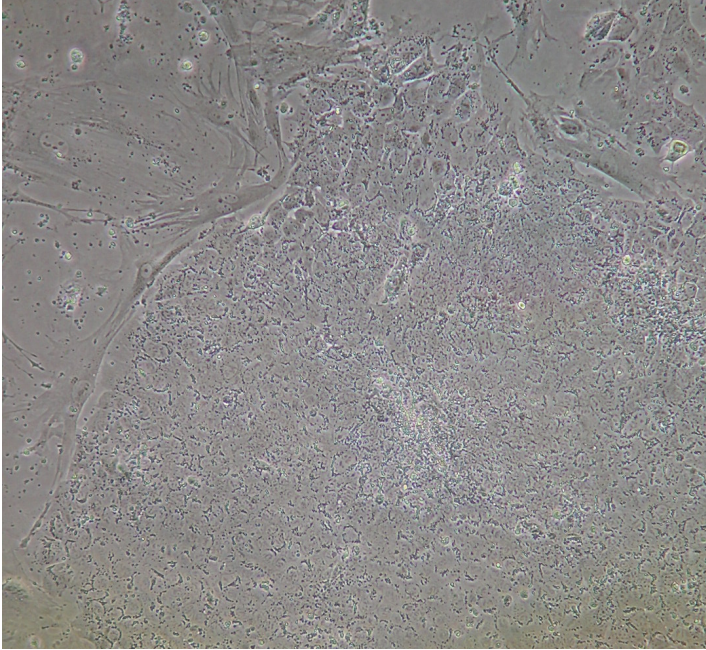
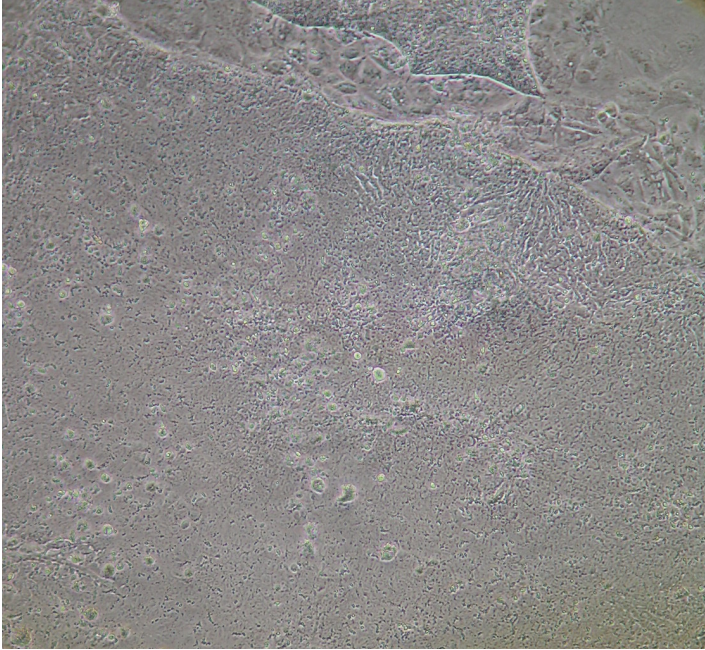
Efficiency of isolation of ES-like Cells

Type of embryos	Number of used Blastocysts	Number of isolated ICM	Number of plated ICM	Number of P1 outgrowths	Number of P2 colonies	Number of obtained passages
Frozen Rabbit blastocysts	644	336 52%	216 64%	54/177 30%	21/47 45%	P3
Fresh Rabbit blastocysts	461	326 71%	280 86%	156/265 59%	107/154 69%	P9
Total Rabbit blastocysts	1105	662 60%	496 75%	210/442 47%	128/201 64%	P3 54% P8 5%
Frozen Goat blastocysts	78	46 59%	40 87%	11/25 44%	1/11 9%	P2
Fresh Goat blastocysts	306	233 76%	161 69%	61/155 39%	16/57 28%	P3
Total Goat blastocysts	384	279 73%	201 72%	72/180 40%	17/68 25%	P3 24%

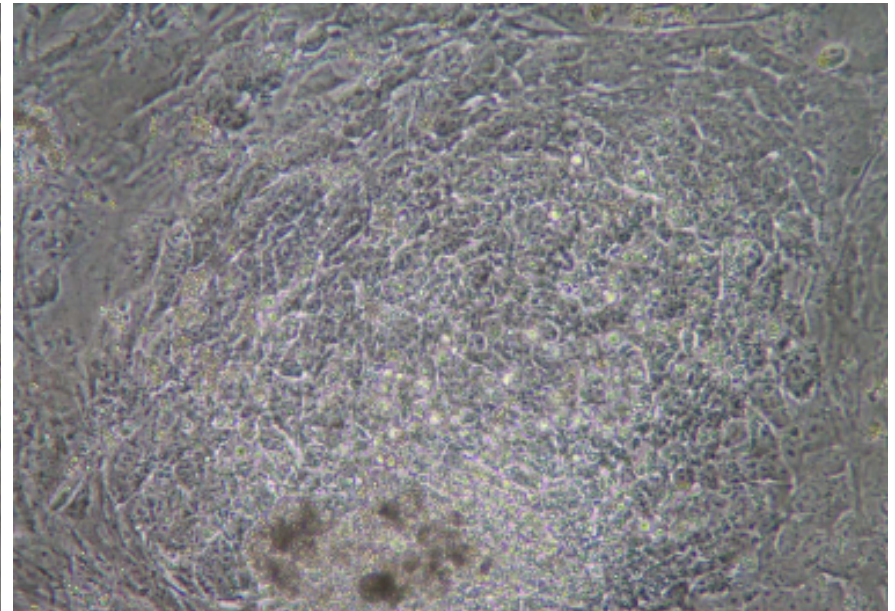
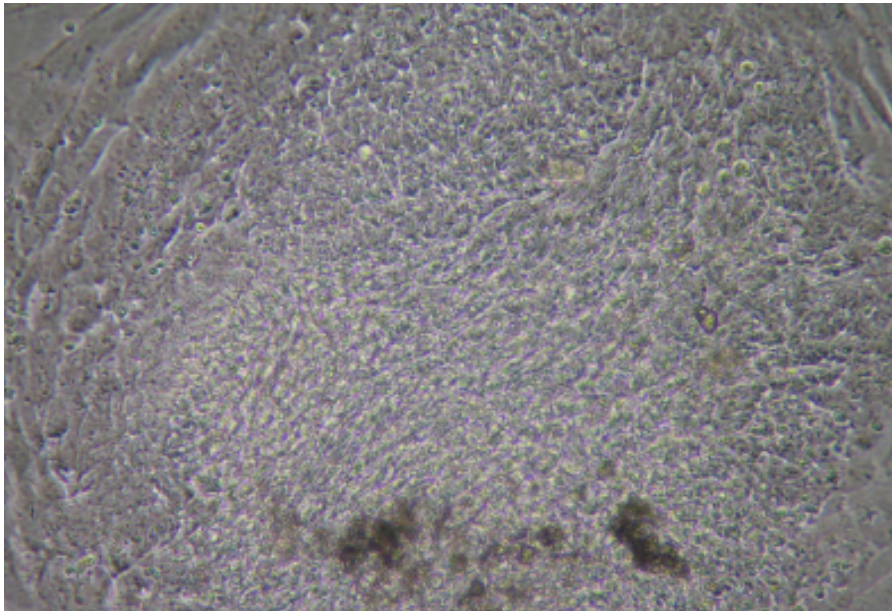
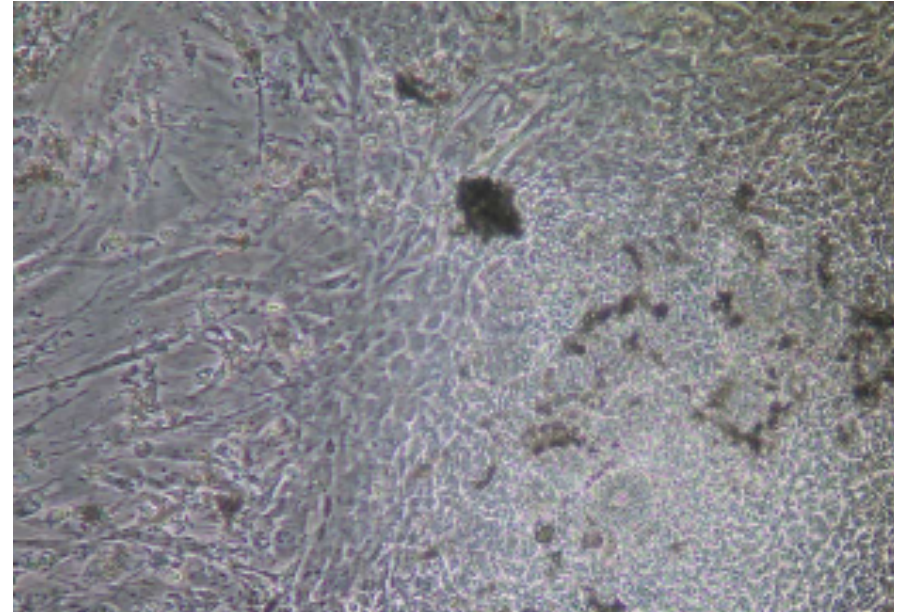
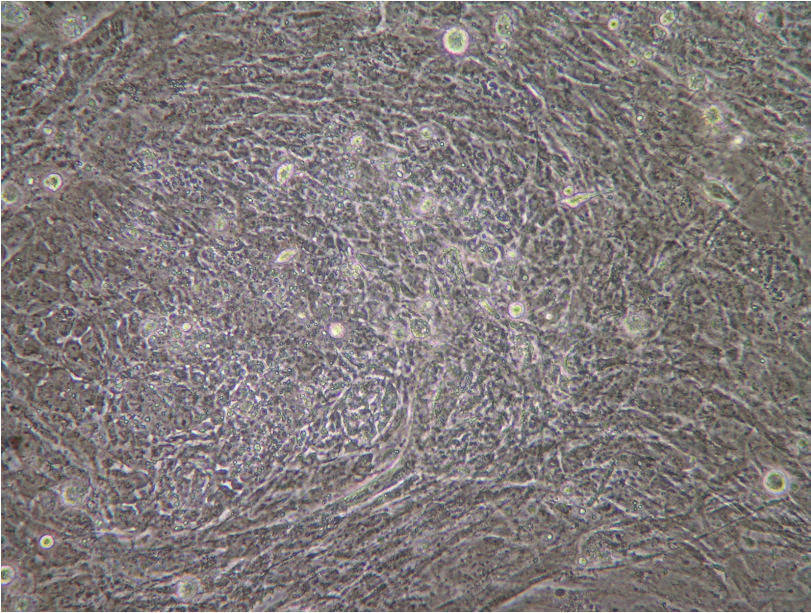
Different morphologies of rabbit P1 outgrowths



Different morphologies of rabbit ES-like P2 colonies



Morphologies of rabbit differentiated colonies

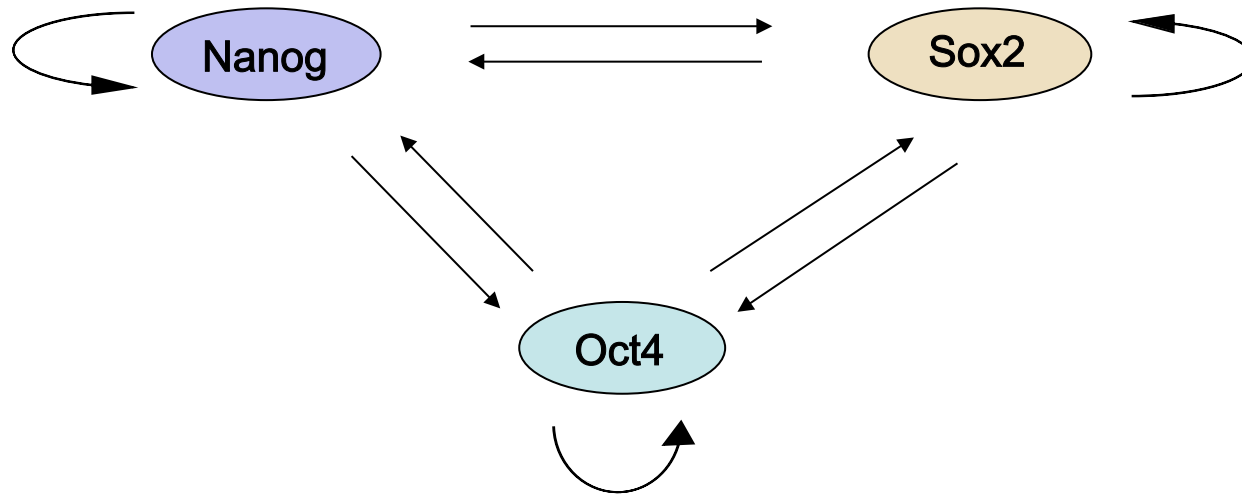


Conclusion 1

- Isolation of Rabbit and Goat ES-like cells with human ES cell features.
- Very low efficiency of ES-like cell isolation.
- Spontaneous differentiation of the ES-like cells after three or eight passages according to the specie.
- A phenomenon associated with the loss of Oct4 expression.
 - ==> Development of strategies to overexpress transcription factors involved in sustaining pluripotency in mouse and human ES cells

Strategies of overexpression of pluripotency genes

Transcription factors involved in sustaining pluripotency in mouse and human ESC



➤ SIV-derived lentiviral vectors

In collaboration with FL Cosset (Inserm U758, ENS Lyon)
Test several amphotropic envelopes
Test different promoters

➤ Tat-mediated protein transduction

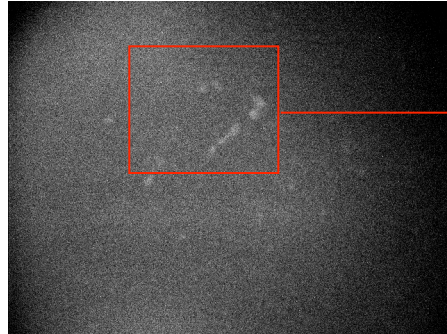
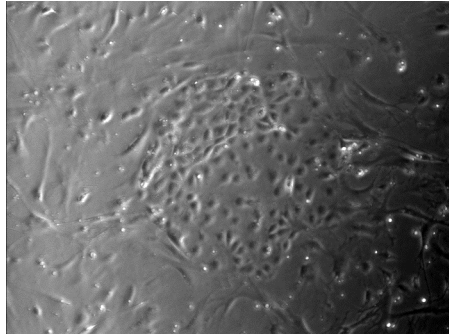
In collaboration with F Edenhofer (Bonn University, Germany)
Test TAT-Nanog protein

Lentiviral infection of rabbit blastocyst cells

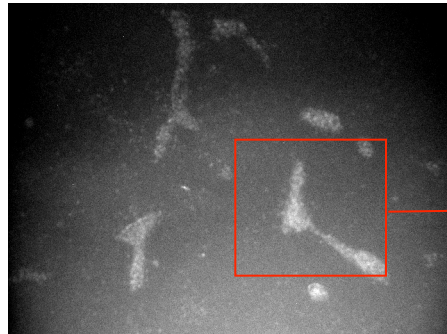
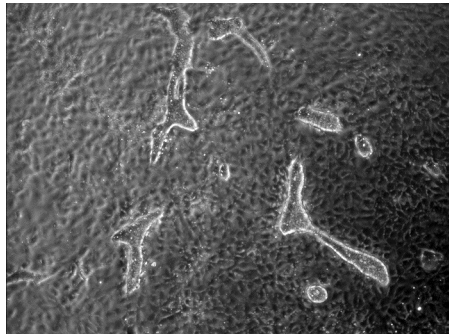
Use of VSV-G pseudotyped vector expressing the GFP gene under the transcriptional control of CAG promoter

Contrast of phase

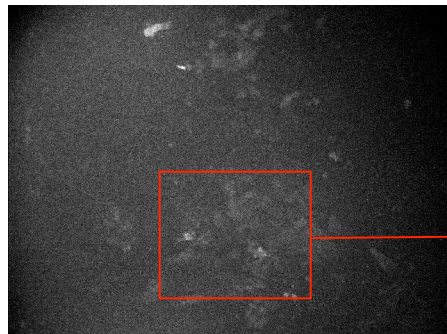
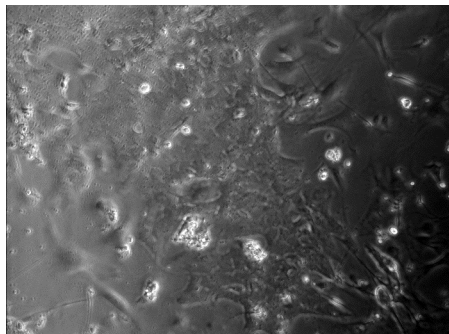
Immunofluorescence



ICM
Passage 1
48h culture



Outgrowths
Passage 1
5 day culture



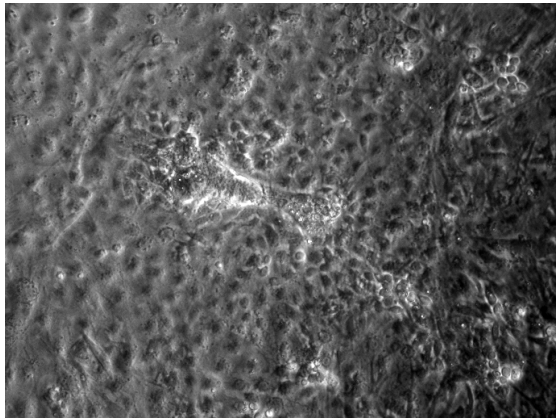
ES-like colony
Passage 2
48h culture

➤ Transgene expression in ES-like cells following lentiviral infection of blastocyst cells

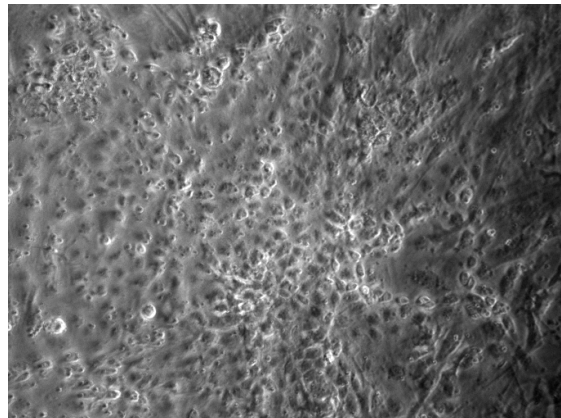
Lentiviral infection of goat blastocyst cells

Use of VSV-G pseudotyped vector expressing the GFP gene under the transcriptional control of CAG or PGK promoters

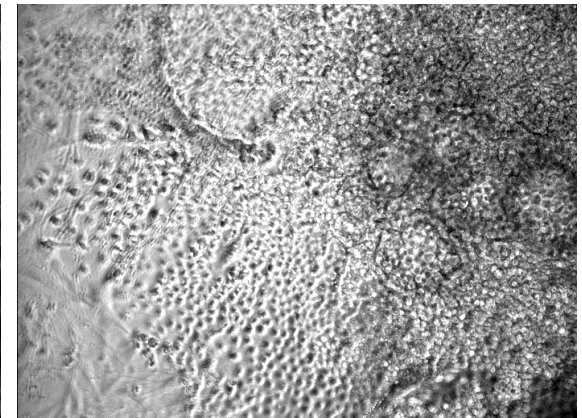
Outgrowths Passage 1
3 day culture



Outgrowths Passage 1
3 day culture



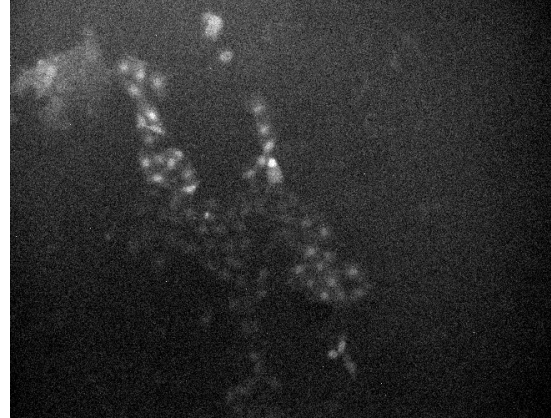
Outgrowths Passage 1
7 day culture



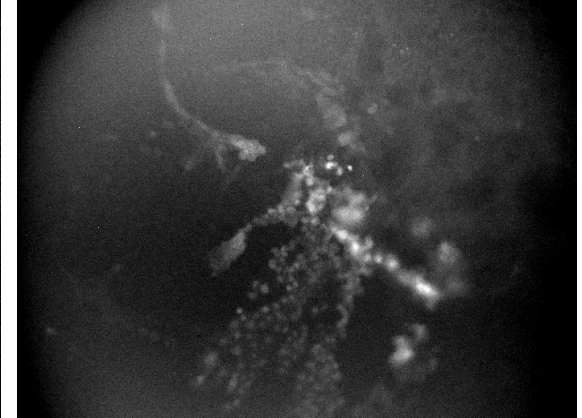
Contrast of phase
Immunofluorescence



CAG promoter



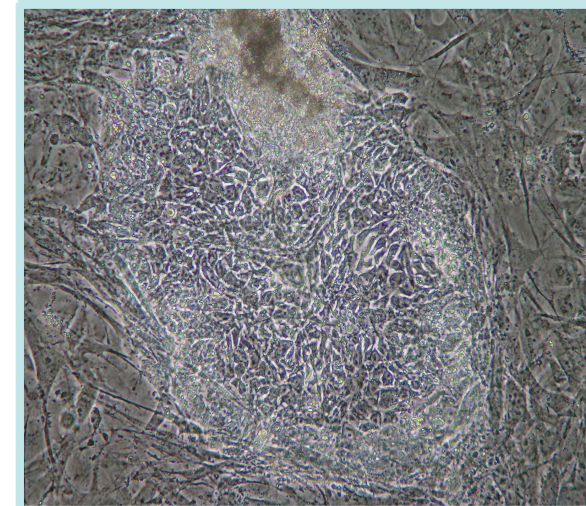
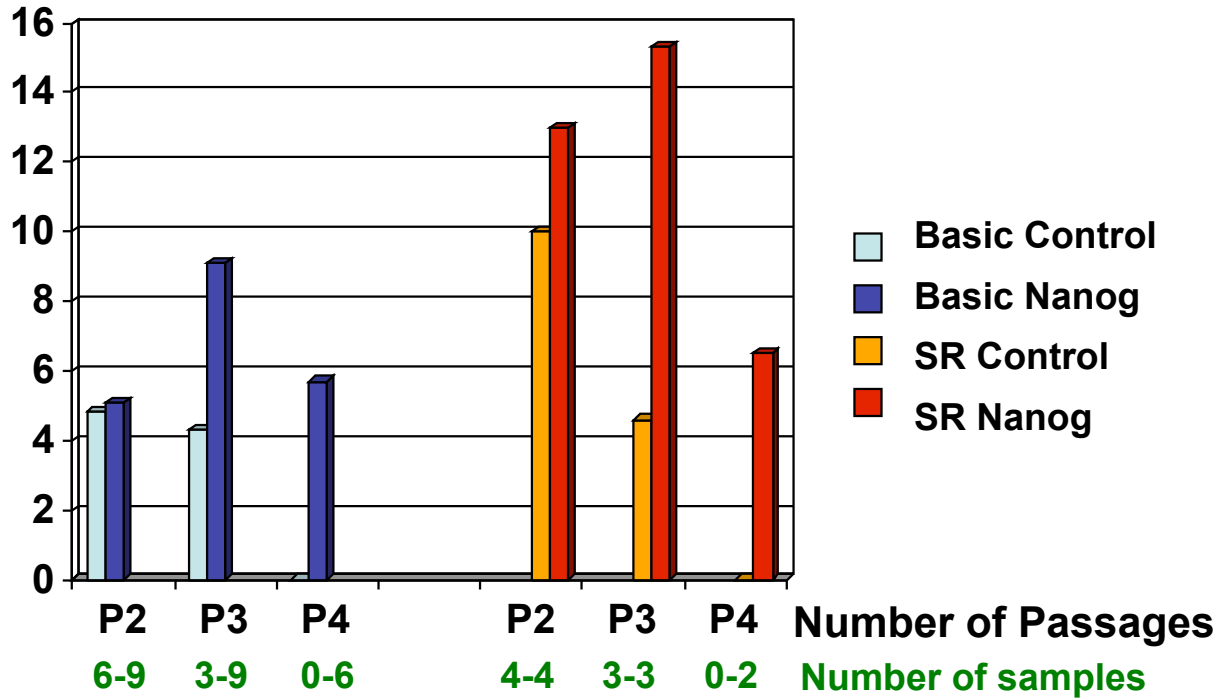
PGK promoter



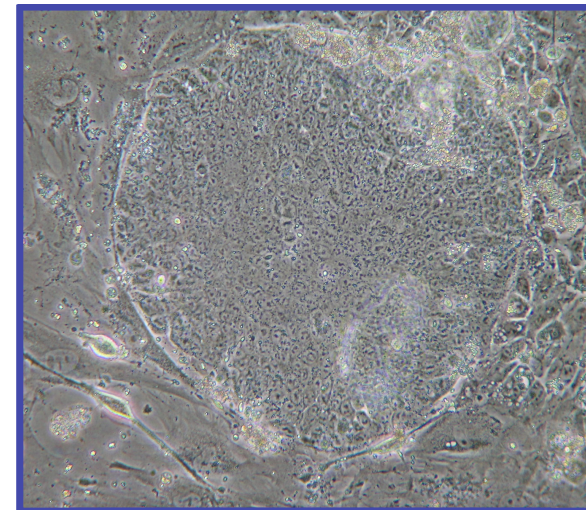
➤ Only PGK promoter induces transgene expression in goat ES-like cells

TAT-Nanog protein transduction of rabbit blastocyst cells

Number of colonies



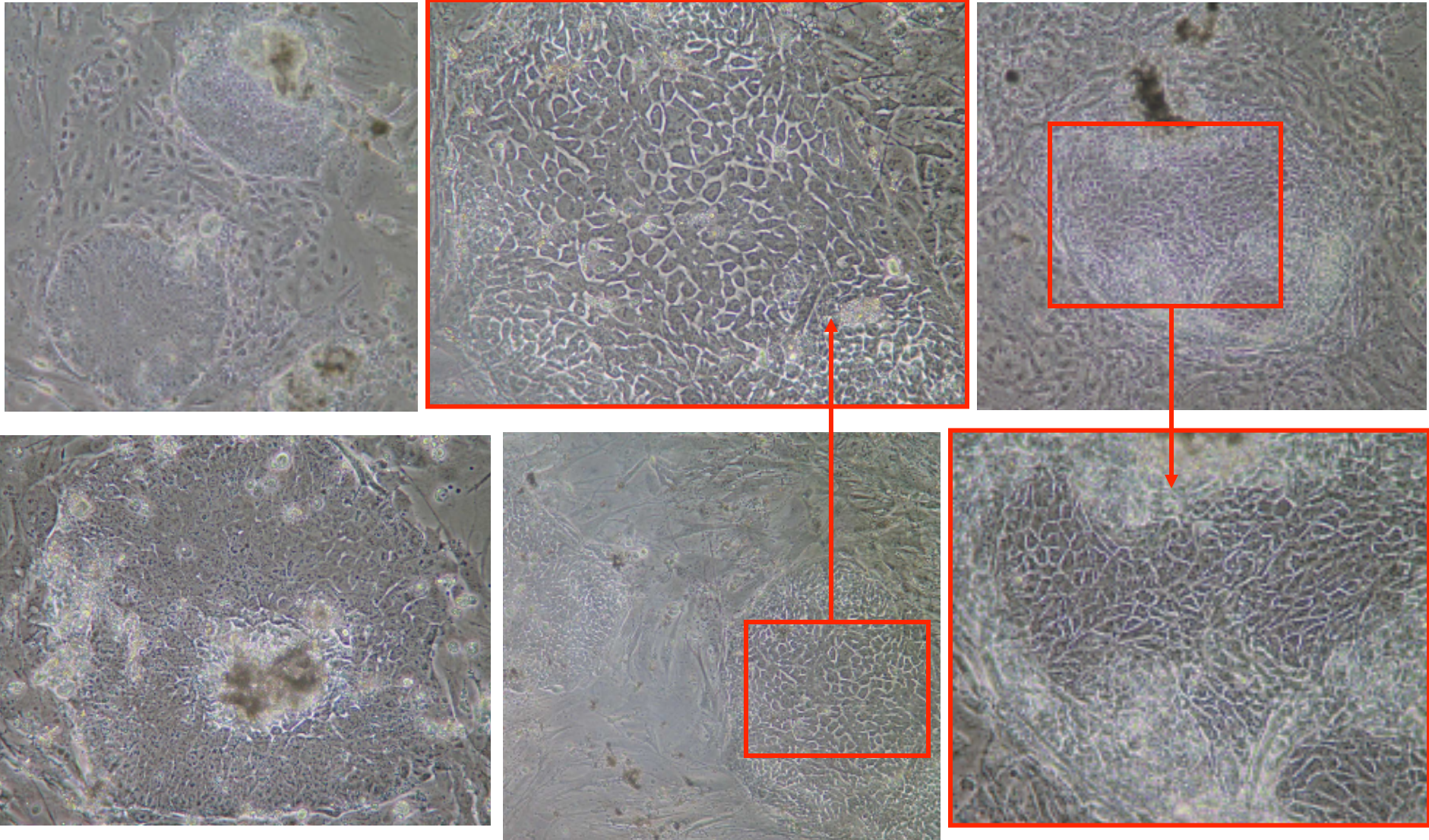
P2 colony
in Basic Control Medium



P2 colony
in Basic Nanog Medium

- TAT-Nanog protein increases the ability of the cells to :
 - give new colonies
 - maintain pluripotency in culture

Morphologies of rabbit TAT-Nanog colonies



Conclusion 2

- **Expression of the GFP reporter gene in rabbit or goat blastocyst cells following lentiviral infection of Inner Cell Mass.**
- **Efficiency of used promoter is different according to the specie: CAG promoter for rabbit cells and PGK promoter for goat cells.**
 - ==> Use of lentiviral vector to overexpress Oct4, Nanog and Sox2 genes in rabbit ES-like cells**
- **TAT-Nanog protein increases the ability of rabbit ES-like cells to maintain pluripotency in culture.**
 - ==> Improvement of the TAT-mediated protein transduction method**



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www.elsevier.com/locate/yexcr



Research Article

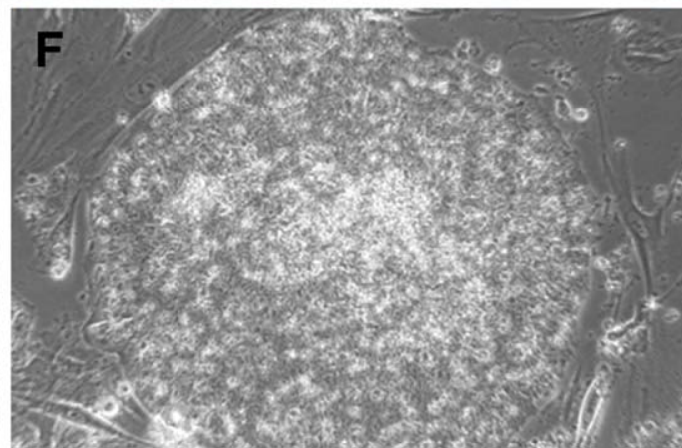
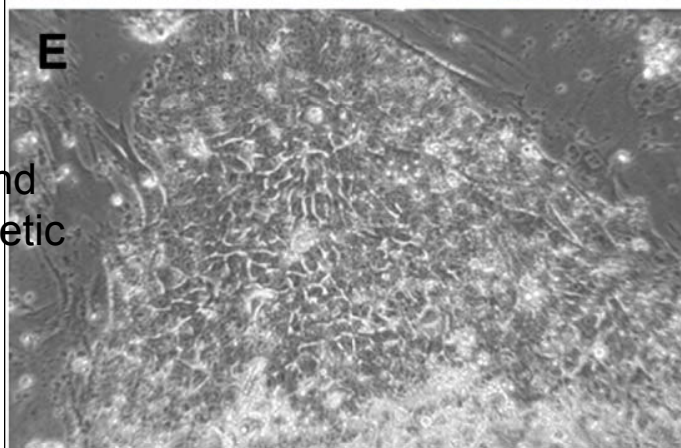
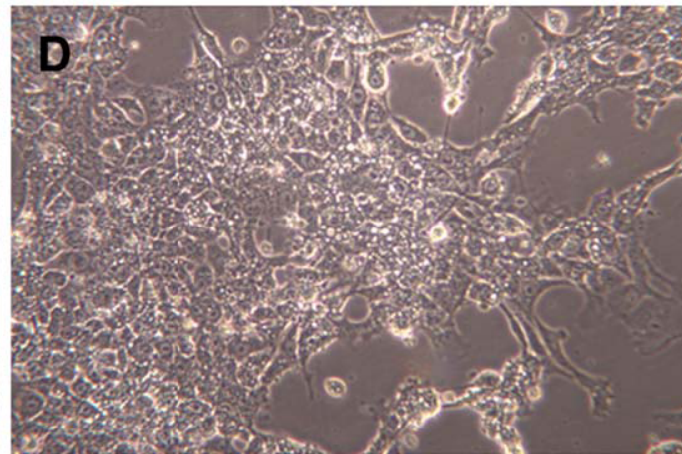
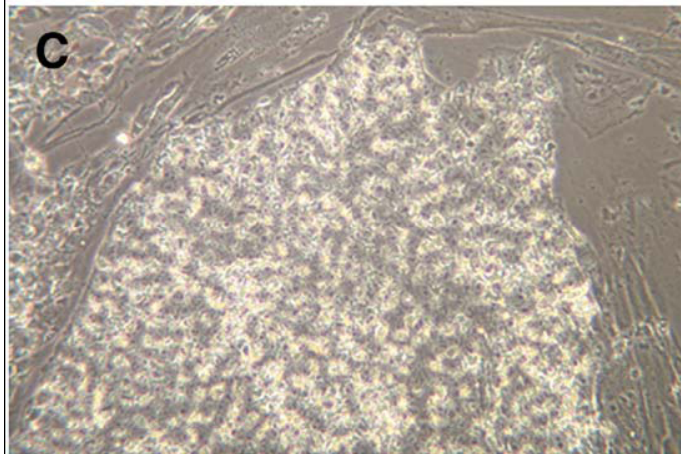
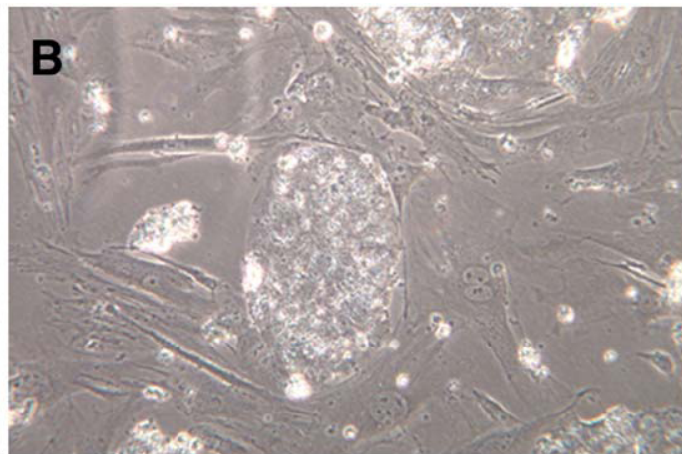
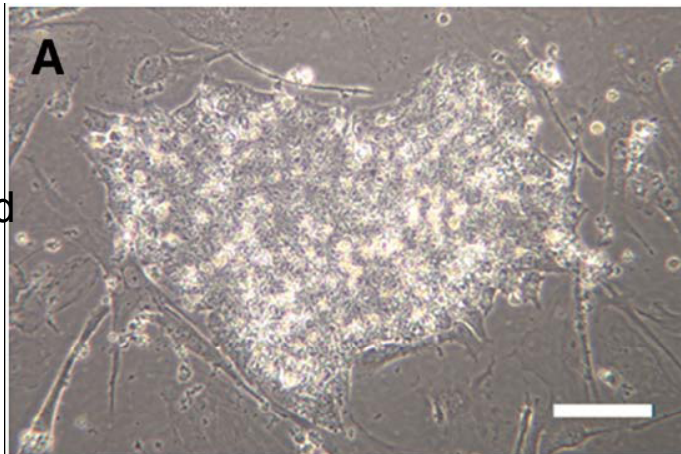
Rabbit embryonic stem cell lines derived from fertilized, parthenogenetic or somatic cell nuclear transfer embryos

Zhen F. Fang¹, Hui Gai^{a,1}, You Z. Huang^{a,b,1}, Shan G. Li^{a,1}, Xue J. Chen^a, Jian J. Shi^{a,b}, Li Wu^a, Ailian Liu^a, Ping Xu^c, Hui Z. Sheng^{a,*}

^aCenter for Developmental Biology, Xinhua Hospital, Shanghai Jiao Tong University, School of Medicine, 1665 Kong Jiang Road, Shanghai 200092, P.R. China

^bInstitute of Biochemistry and Cell Biology, Shanghai Institute for Biology Sciences, Chinese Academy of Science, Shanghai 200092, P.R. China

^cShanghai Laboratory Animal Center, Chinese Academy of Science, Shanghai 201615, P.R. China



New Zealand
fertilized
embryos

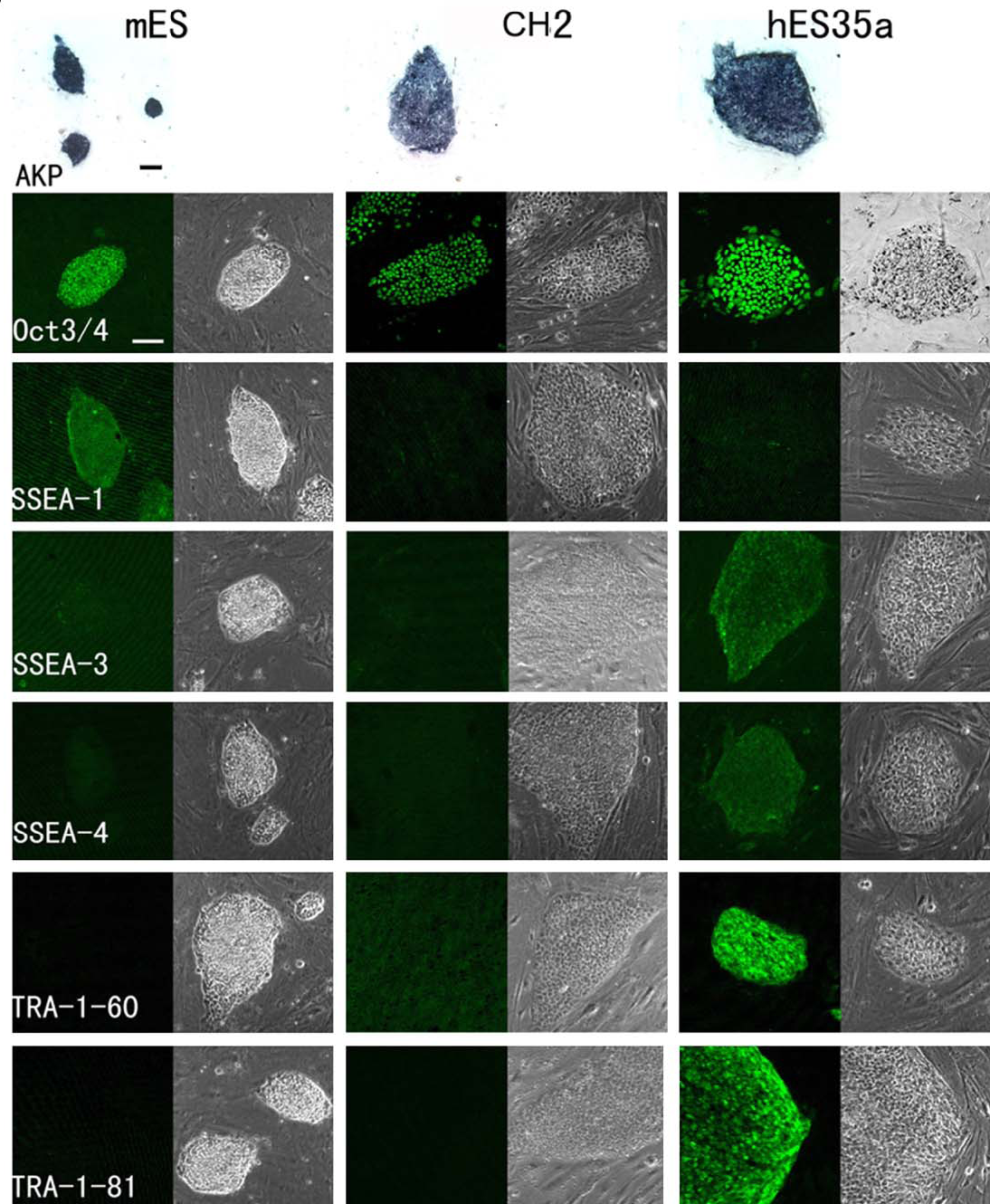
Chinchilla
fertilized
embryos

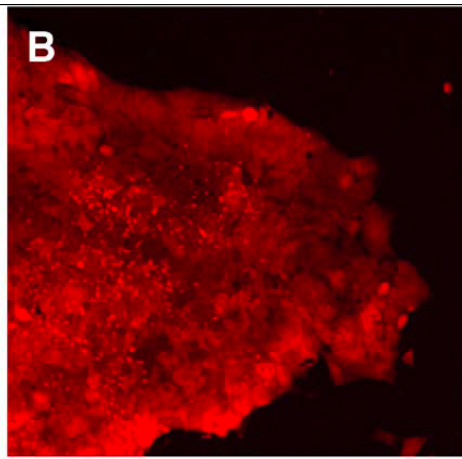
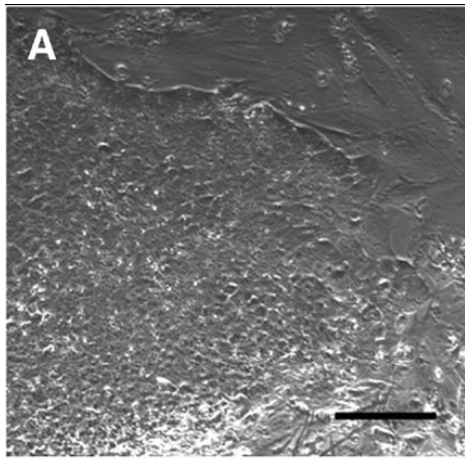
Angora
fertilized
embryos

Differentiation
without
feeders

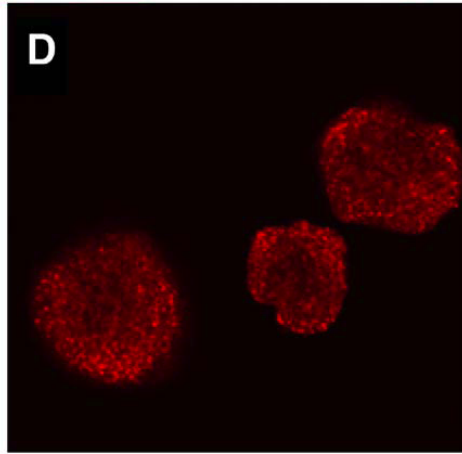
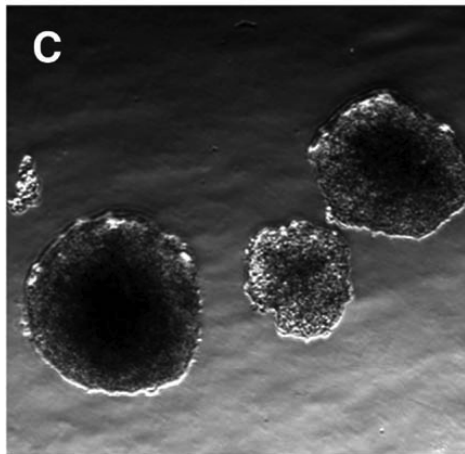
New Zealand
parthenogenetic
embryos

New Zealand
nuclear
transfer
embryos

B



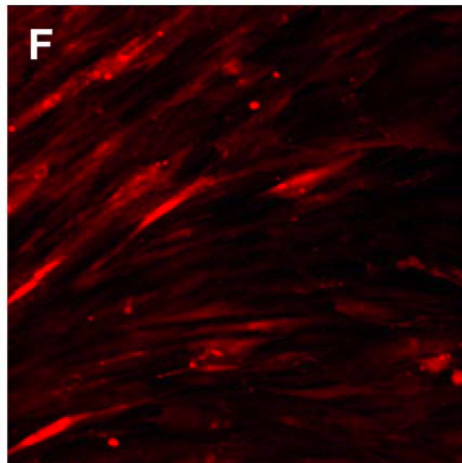
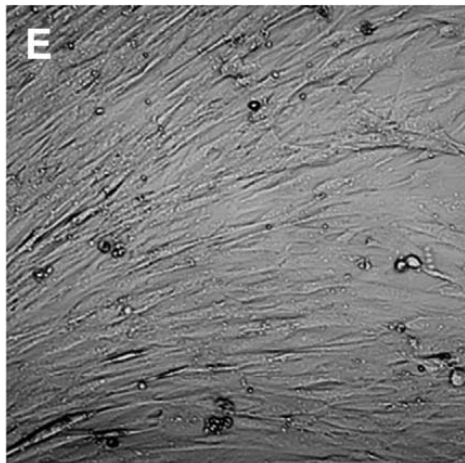
New Zealand RFP ES Cells



RFP
EB



Nuclear transfer rabbit :
Chinchilla ES cells in
New Zealand white background



Differentiated RFP ES Cells

Test Chinese ES cell Medium

Comparison of two media :

French ES medium : KO-DMEM + 10% FBS + 10% SR + 8 ng/ml FGF2

Chinese F12 medium: DMEM/F12 + 20% SR + 8 ng/ml FGF2

Results :

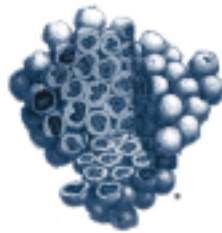
Immunosurgery of 347 blastocysts ==> culture of 270 ICM (78%)

Medium	F12 medium	ES medium
Plated ICM	133	123
P1 with outgrowths	91 (68%)	64 (52%)
P2 with ES-like colonies	31 (34%)	30 (47%)
P3 with ES-like colonies	0 (0%)	6 (20%)

F12 medium gives more and nicer P1 outgrowths and more P2 ES-like colonies
ES medium allows to obtain less ES-like colonies but nicer and until passage 4

Conclusion :

==> F12 medium does not amplify or maintain in culture our ES-like cells



STEM CELLS[®]

Generation and Characterization of Rabbit Embryonic Stem Cells

Shufen Wang, Xianghui Tang, Yuyu Niu, Hongwei Chen, Bin Li, Tianqing Li, Xiuzhen Zhang, Zhixin Hu and Weizhi Ji

Stem Cells published online Oct 12, 2006;

DOI: 10.1634/stemcells.2006-0226

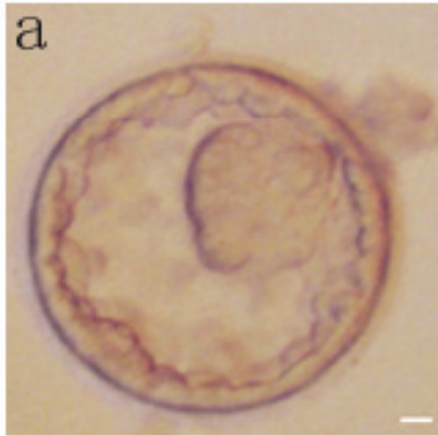
This information is current as of October 15, 2006

The online version of this article, along with updated information and services, is located on the World Wide Web at:

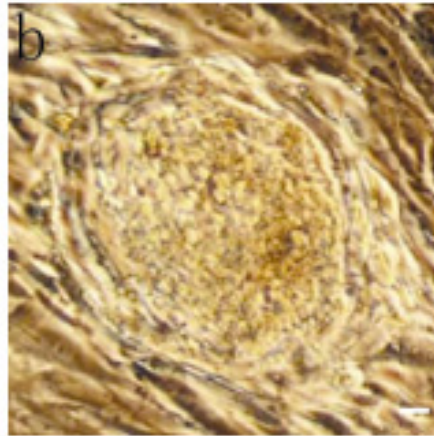
<http://www.StemCells.com>

Fertilized embryo

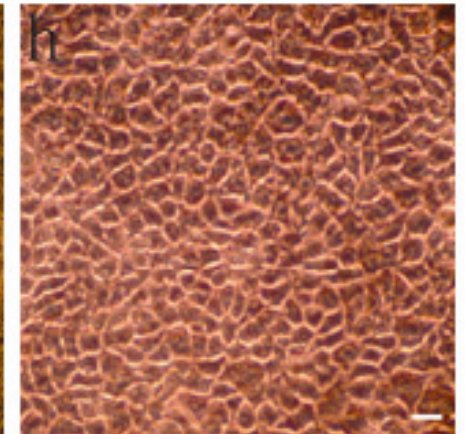
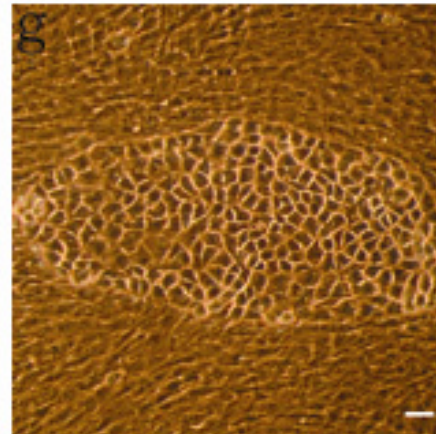
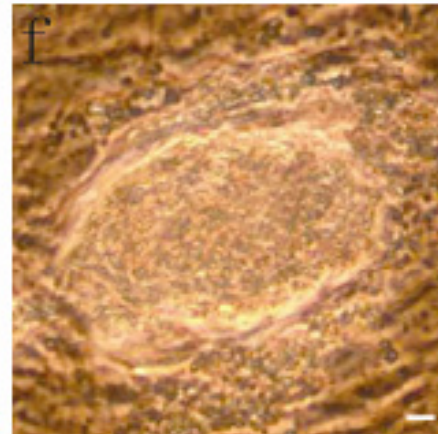
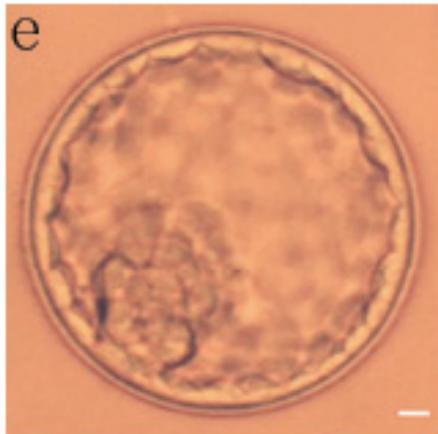
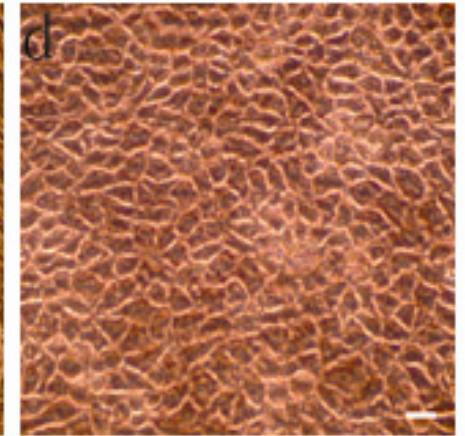
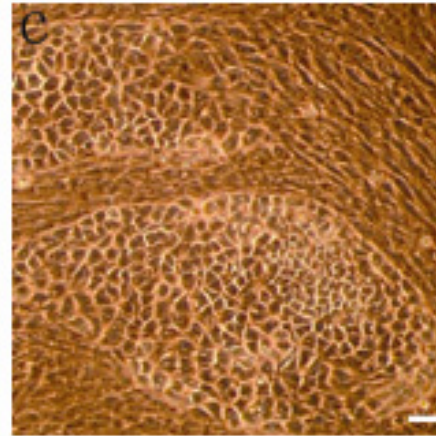
Blastocyste



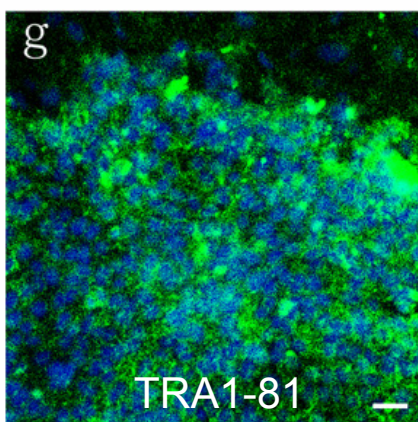
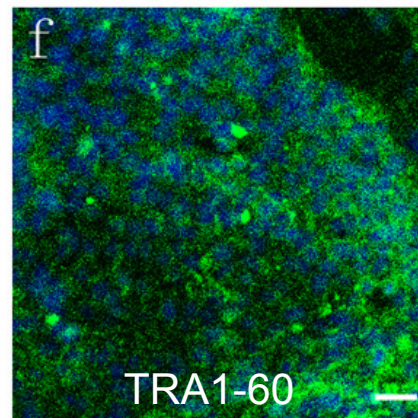
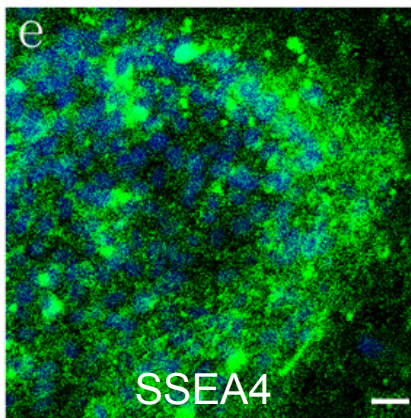
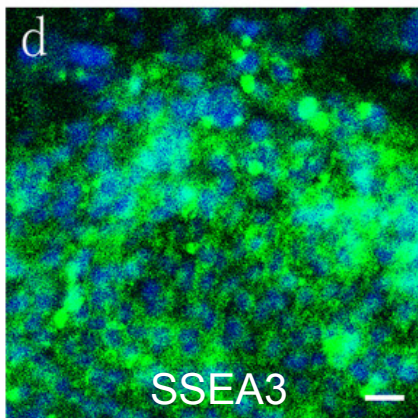
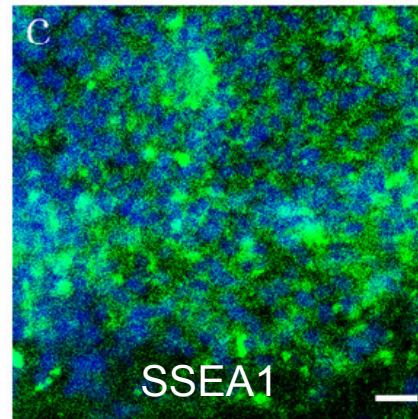
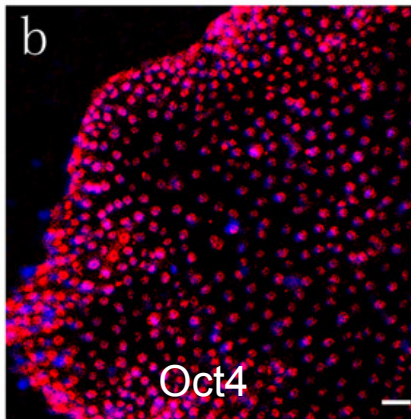
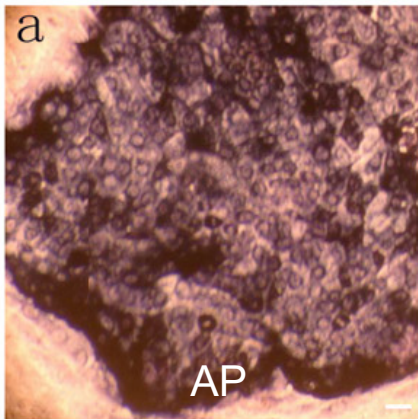
4 days ICM outgrowth



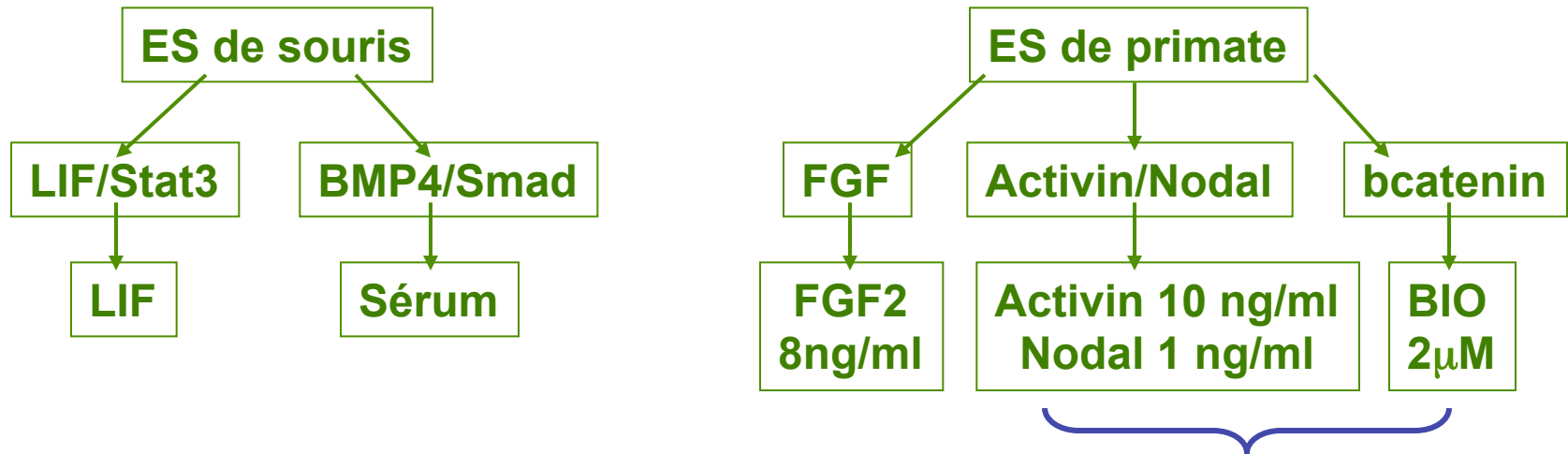
ES cell colonies



Parthenogenetic embryo



Test effect of Activin/Nodal/BIO on ES-like cell culture



Comparison of Chinese F12 and French ES media +/- factors

Results :

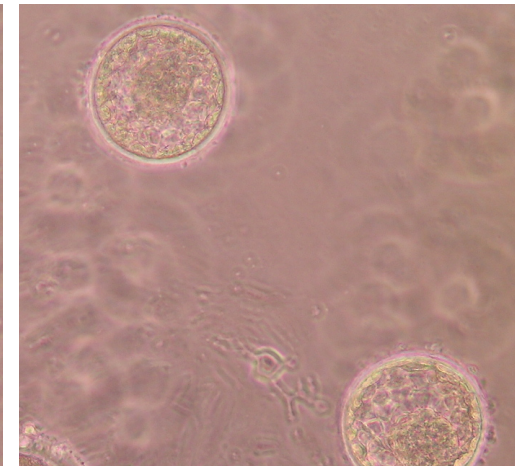
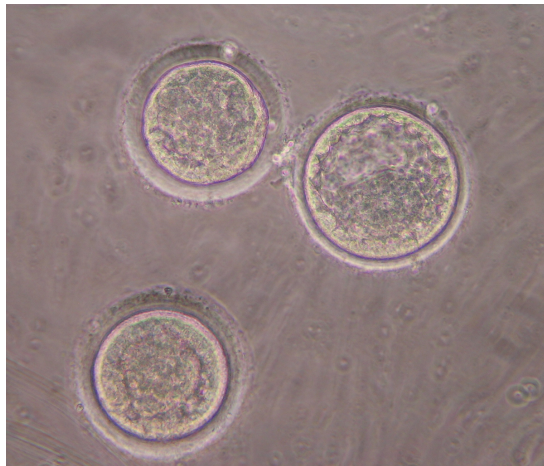
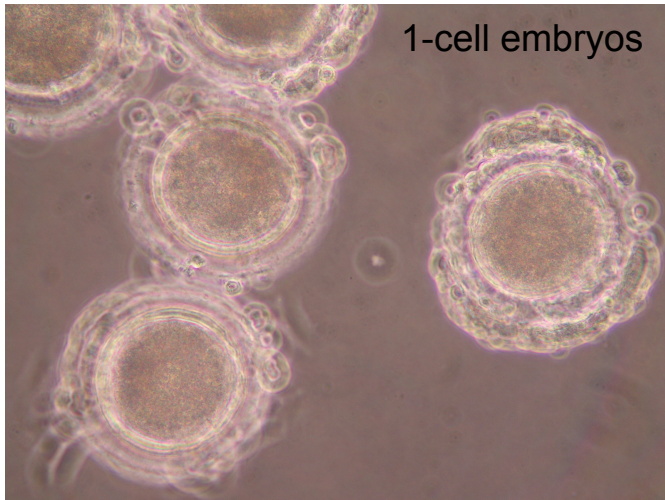
Immunosurgery of 141 blastocysts
 ==> culture of 102 ICM
 (21 ICM in each medium without factors and 30 ICM in each medium with factors)

Conclusion :

==> Addition of factors (Activin, Nodal and BIO) is not sufficient to maintain in culture our ES-like cells.

Medium	ES	ES + Factors	F12	F12 + Factors
Plated ICM	21	28	16	30
P1 with outgrowths	12 (57%)	28 (46%)	10 (62%)	15 (50%)
P2 with ES-like colonies	10 (83%)	12 (92%)	4 (40%)	9 (60%)
P3 with ES-like colonies	6 (60%)	1 (8%)	3 (75%)	0 (0%)

Test culture of embryos

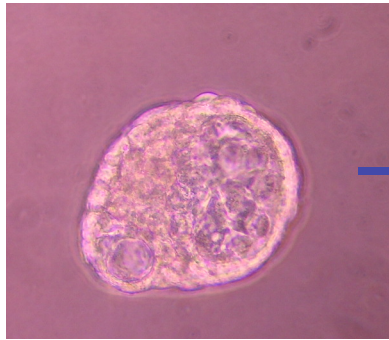


Results:

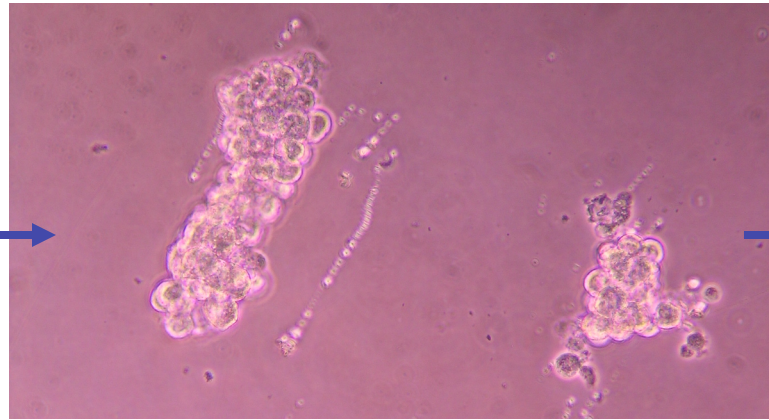
==> Culture of 132 1-cell embryos ==> 111 developed blastocysts (84%)

==> **Development rate of blastocysts in culture is correct**

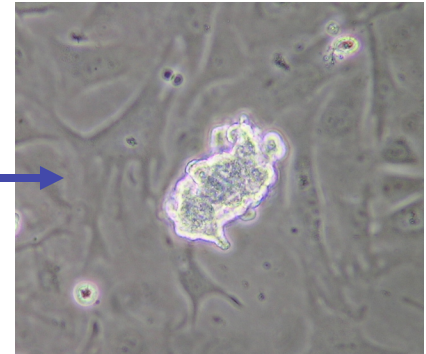
Test isolation of ICM with trypsin



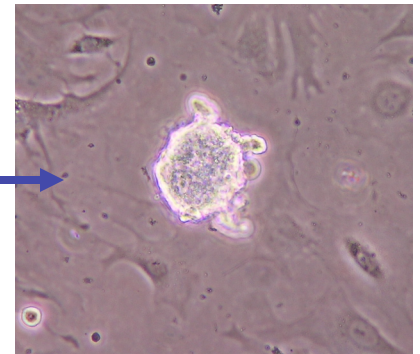
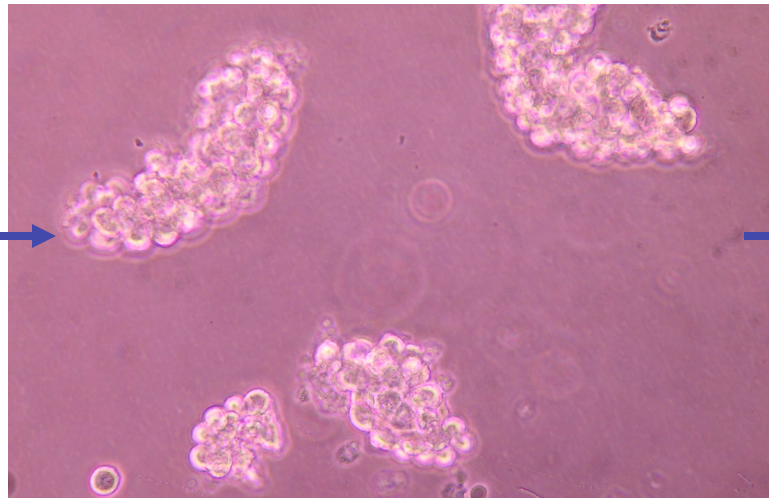
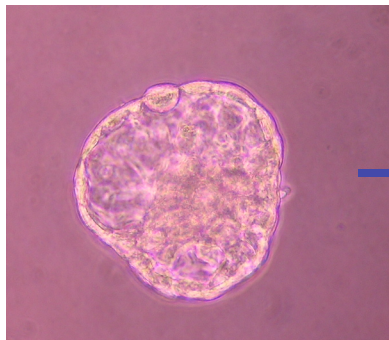
Blastocyst after trypsin



Blastocyst after mechanical dissociation



ICM on feeder Cells



Results:

84 blastocysts from 1-cell culture ==> 59 ICM after Trypsin (70%)

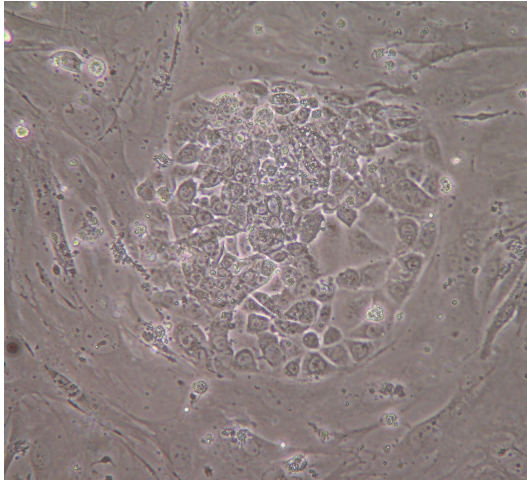
78 blastocysts from thawed morula ==> 42 ICM after Trypsin (54%)

Comparison of Trypsin with Immunosurgery :

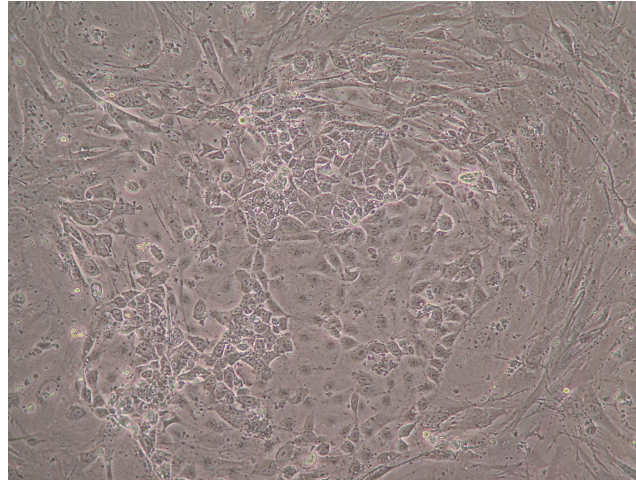
==> quicker but ICM are less visible and could be easily damaged by trypsin

Test medium used by Shufen Wang

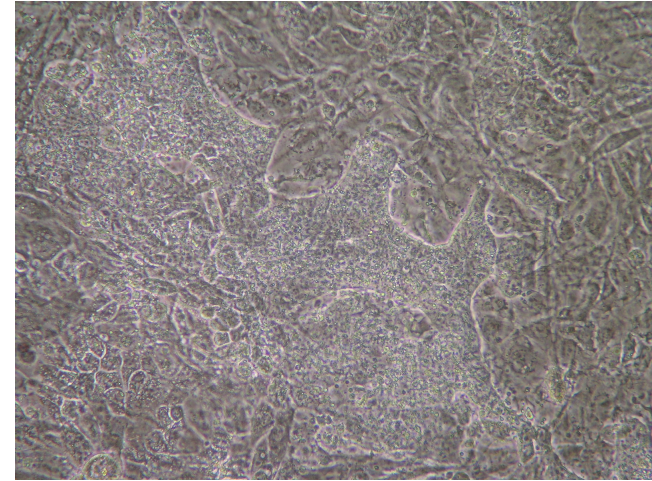
Plated ICM after 48h



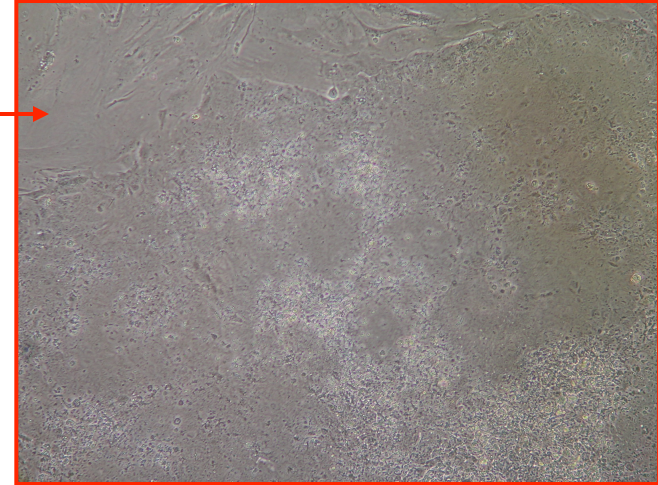
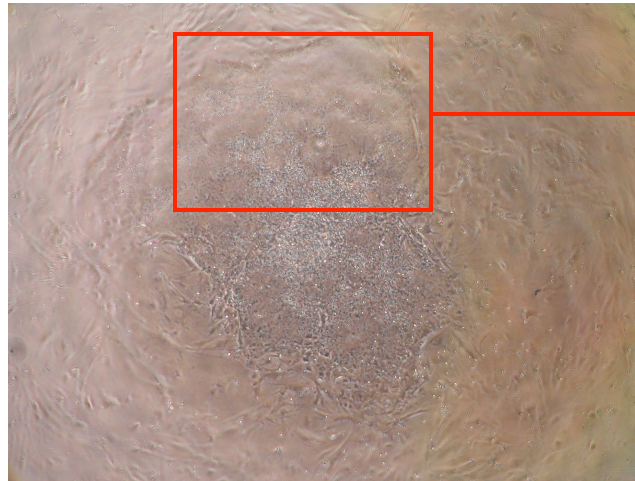
Plated ICM after 3 days



P1 outgrowths after 6 days



ES-like
P2 colony



Results:

Used medium: DMEM + 15% FBS + 1% β ME + 1% NEAA + 1% PS + 1,5% G

Test on 101 ICM isolated from trypsinized blastocysts

Appearance of less extended and flatter P1 outgrowths but usual P2 ES-like colonies

Conclusion:

==> This medium does not maintain by himself the self-renewal of our ES-like cells

Changing type and concentration of feeder cells

Comparison of 129 MEF and CF1 MEF:

Immunosurgery of 103 blastocysts ==> culture of 60 ICM

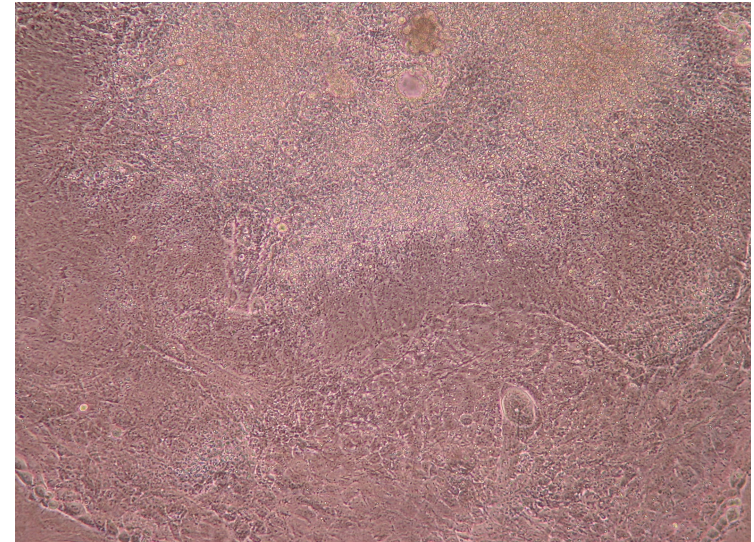
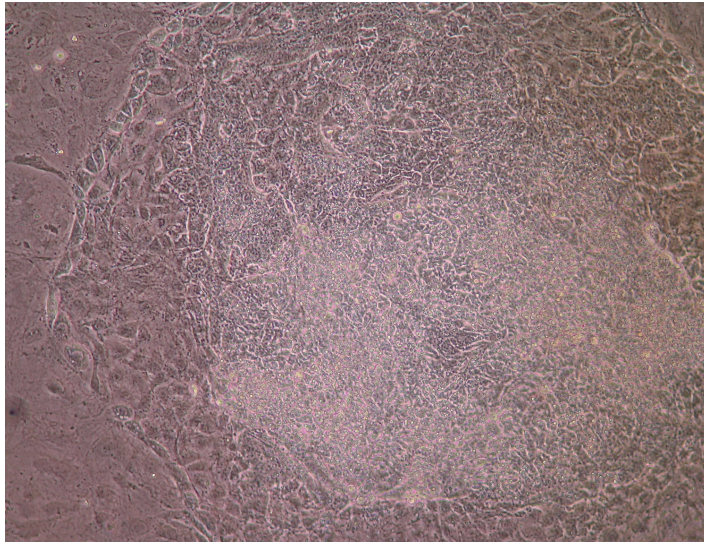
==> No difference between the two types of feeder cells

Type of feeder	Plated ICM	P1 outgrowths	P2 with ES-like colonies
129 MEF	29 (100%)	15 (51%)	11 (37%)
CF1 MEF	31 (100%)	17 (55%)	8 (26%)

Test higher concentration of feeder cells:

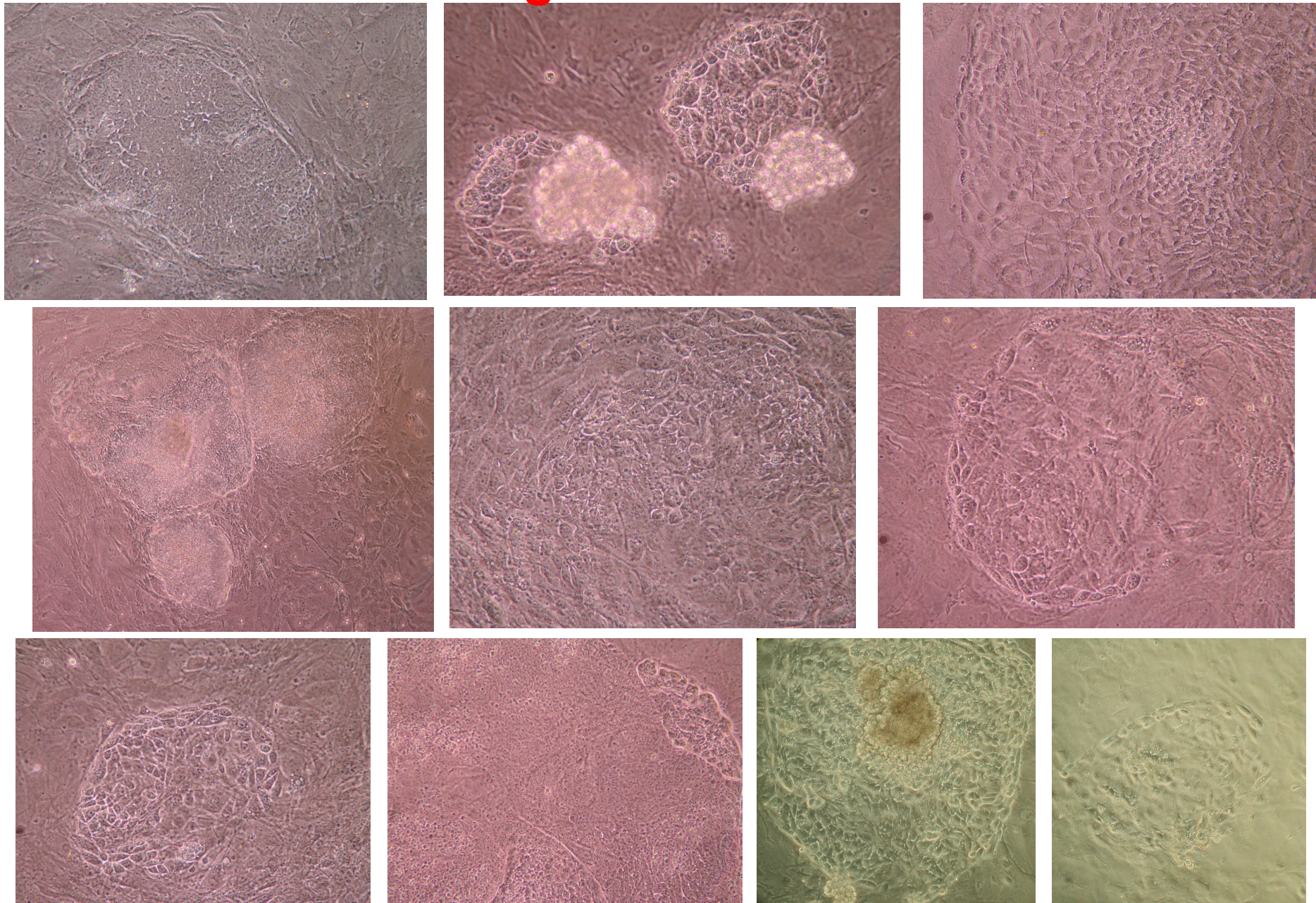
4 times more : 1.5×10^5 cells/well (usually 4×10^4 cells/well, with 4-well plate)

==> Appearance of unusual P1 outgrowths



==> Appearance of different types of P2 colonies

P2 colonies on high concentrated feeder cells



==> Concentration of feeder cells seems to be the most important factor to derive rabbit ES cells

Conclusion 3

- The medium do not seem to be essential
- The culture of embryos could be important
- The quality and the density of feeder cells is crucial
 - ==> Use of Shufen's method for rabbit ESC derivation
 - ==> Use of Shufen's rabbit ESC as nuclear donor cell for cloning rabbit in the laboratory of Jean-Paul Renard

Thanks to...



**Henry
Kennedy**



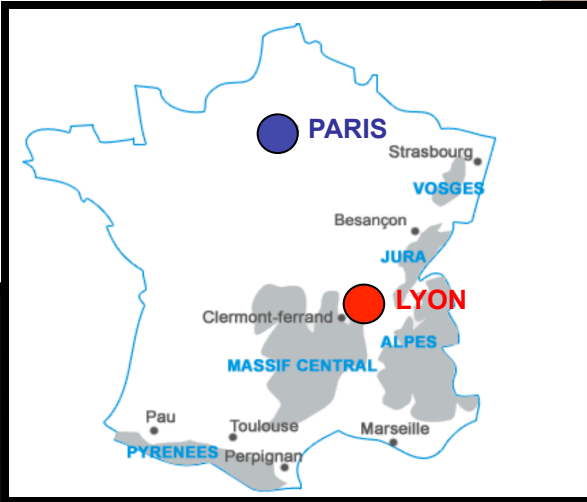
**Pierre
Savatier**



**Suzy
Markossian**



**Florence
Wianny**



Welcome in France!
Welcome in Lyon!

