Instituto de Salud Carlos III

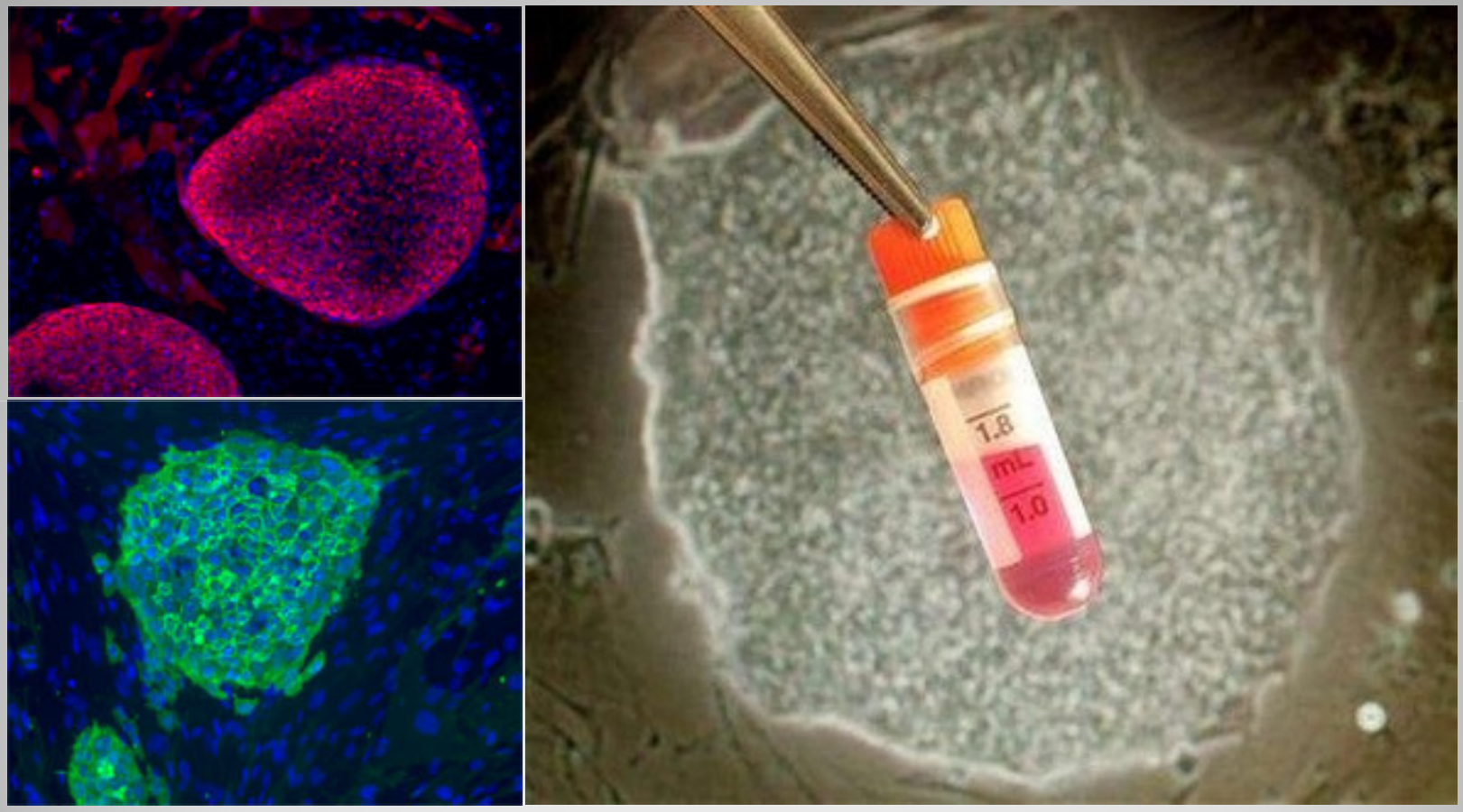
PRINCIPE FELIPE
CENTRO DE INVESTIGACION

Freezing and thawing of pluripotent stem cells

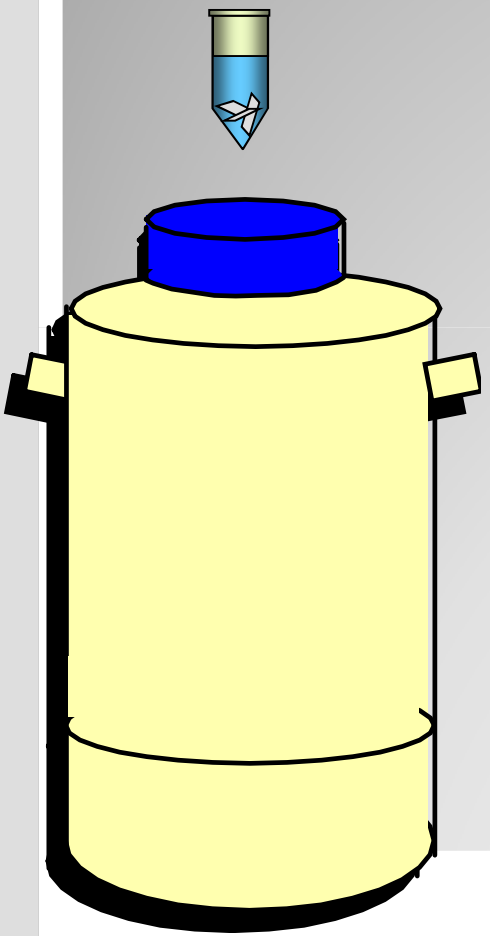
Diana Valbuena Perilla

Spanish Stem Cell Bank-Valencia Node

Human embryonic stem cells



Banking hESC



Background

- Embryo freezing (Lassalle et al, *Fertil Steril* 1985)
- Blastocyst vitrification (Vanderzwalmen, *Human Reprod* 1997)
- Oocyte vitrification (Kuwayama et al, *Reprod Biomed Online* 2005)
- Blastomere cryopreservation (Leveroni, *Cryobiology* 1998, Strussman, 1999, Kusuda,2002)

hESC cryopreservation

- Slow-rate freezing, rapid thawing (Freshney, 1994)
- DMSO 10% + FBS 90%
- Survival rates: <15%
- High level of differentiation and cell death
- 2 weeks to start prolifferation after thawed

Vitrification protocolos

Human Reproduction Vol.16, No.10 pp. 2187–2194, 2001

Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method

B.E.Reubinoff[1,3], M.F.Pera[2], G.Vajta[2] and A.O.Trounson[2]

-Comparison of slow conventional method (FCS 90%) and OPS (20% FBS), DMSO, EG and sucrose
-HES-1 and HES-2 lines

Vitrification protocolos

Table I. Characteristics of human embryonic stem (ES) cell colonies that were recovered from vitrification, in comparison with non-vitrified control colonies

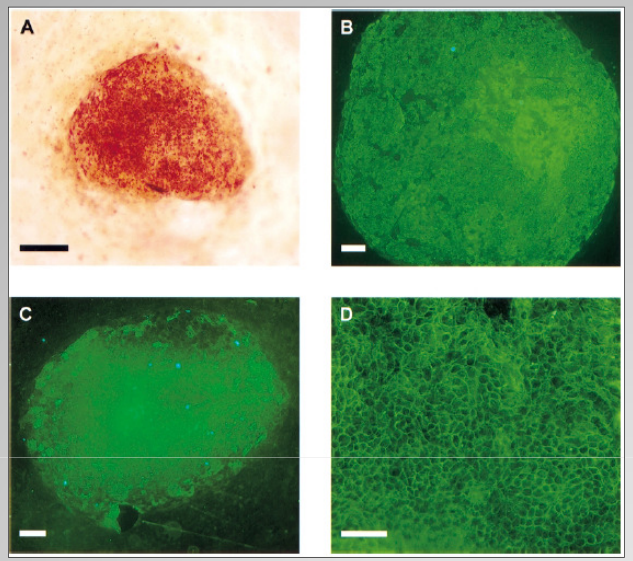
	Control colonies Day 2 (n = 26)	Vitrified colonies Day 2 (n = 25)	P^*	Control colonies Day 7 (n = 26)	Vitrified colonies Day 7 (n = 25)	P^+	Vitrified colonies Day 8 (n = 25)	P^+	Vitrified colonies Day 9 (n = 25)	P^+
Area of colonies (mm^2)[a]	0.59 ± 0.14	0.28 ± 0.08	< 0.001	4.42 ± 0.78	2.63 ± 0.82	< 0.001	4.1 ± 1.31	0.3	5.6±1.7	< 0.004
No. of colonies										
Mainly undifferentiated	–	–	–	12(46)	6(24)	< 0.003	8(32)	0.048	8(32)	0.082
Mainly differentiated	–	–	–	14(54)	13(52)	–	13(52)	–	14(56)	–
Completely differentiated	–	–	–	0(0)	6(24)	–	4(16)	–	3(12)	–

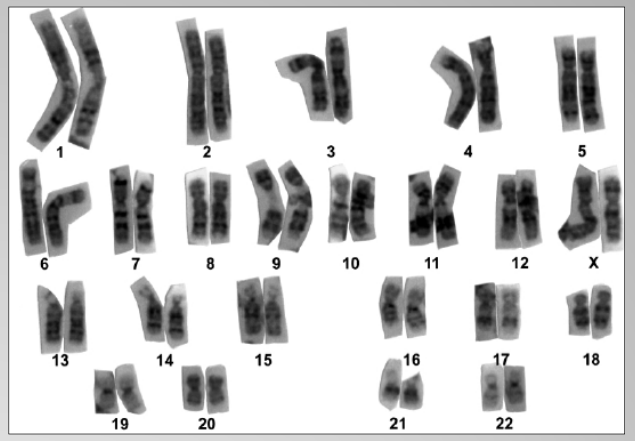
Values are mean ± SD.
*Student's t-test comparison between control and vitrified colonies at day 2.
+Student's t-test and χ^2 test, comparisons between control colonies at day 7 and colonies from vitrified cells at days 7, 8 and 9.
Values in parentheses are percentages.

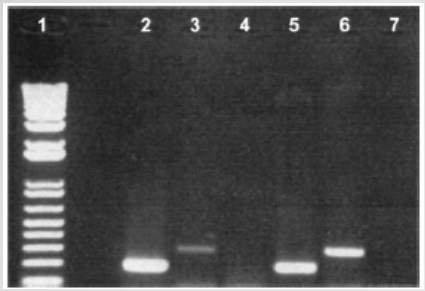
-Better survival rate achieved
-Differentiation still an issue

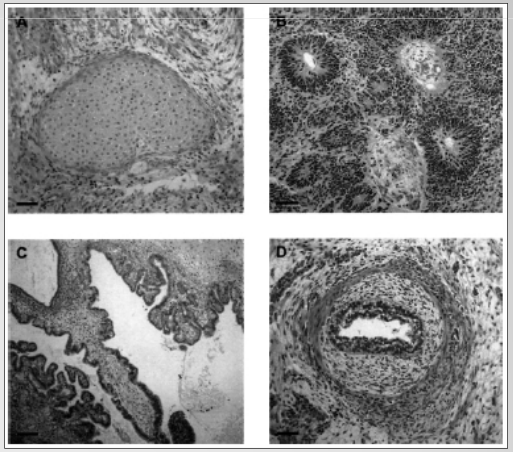
Reubinoff et al, 2001

Vitrification protocolos





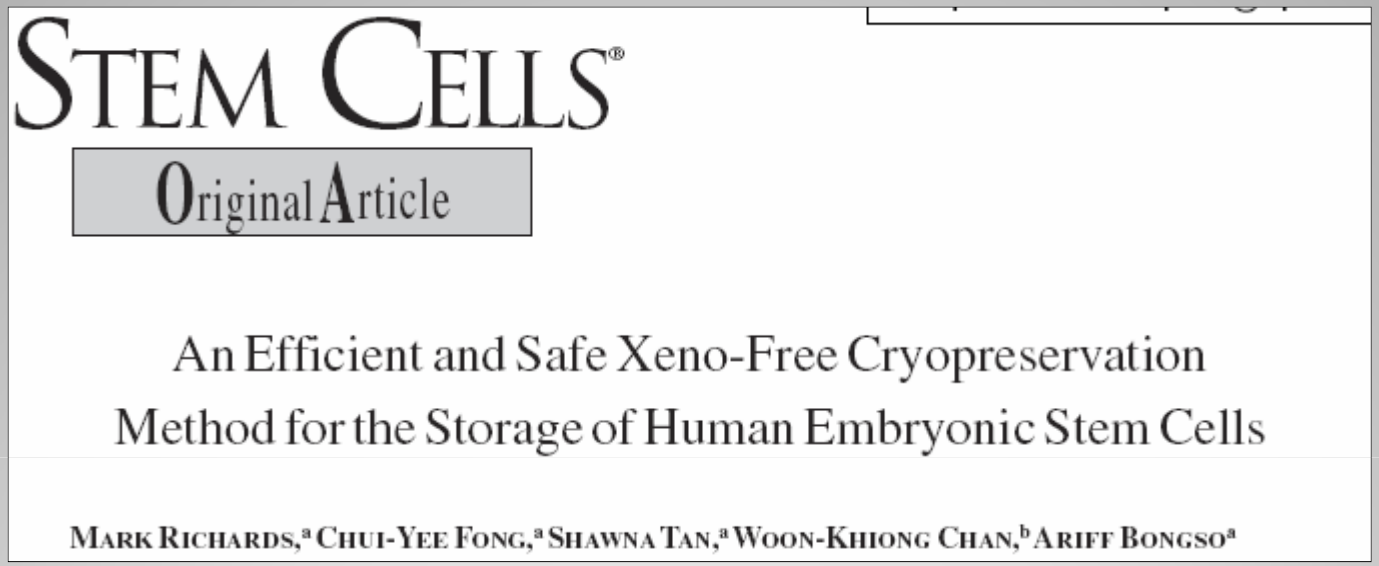




Reubinoff et al, 2001

-Quick and rapid and less toxicity
-Small quantity of hESC colonies (4-6)

Vitrification protocolos



STEM CELLS®

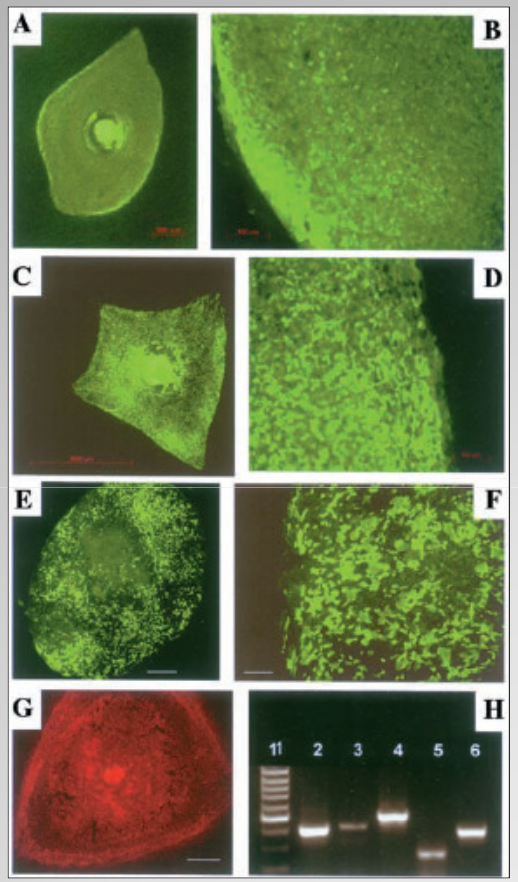
Original Article

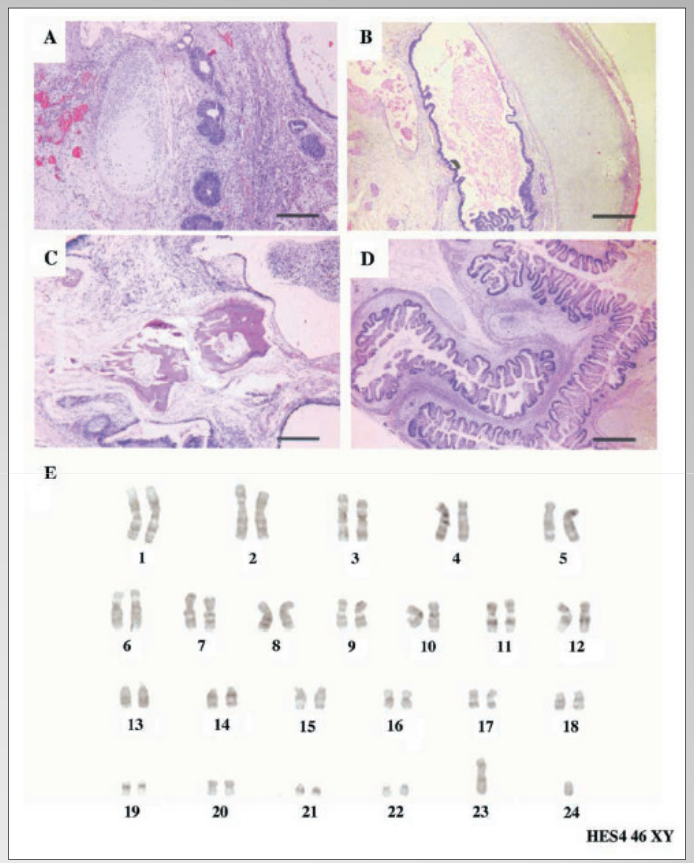
An Efficient and Safe Xeno-Free Cryopreservation Method for the Storage of Human Embryonic Stem Cells

MARK RICHARDS,[a] CHUI-YEE FONG,[a] SHAWNA TAN,[a] WOON-KHIONG CHAN,[b] ARIFF BONGSO[a]

-Comparison of slow conventional method (FCS 90%), OPS (20% FBS) and CS (EG and sucrose), in VLN_2 or LLN_2 and HSA vrs FCS
-HES-3 and HES-4

Vitrification protocolos





Richards et al, 2004

Vitrification protocolos

Table 1. Mean ± standard error of the mean percent post-thaw survival and differentiation of human embryonic stem cells (hESCs) after cryopreservation using different protocols

	Control	Experimental						
hESC colonies	OPS-FCS-LLN_2	CS-HSA-VLN_2	CS-HSA-LLN_2	CS-FCS-LLN_2	CS-FCS-VLN_2	OPS-FCS-VLN_2	CV-FCS-LLN_2	CV-FCS-VLN_2
Grade A	52.3 ± 5.5	58.8 ± 3.0	47.6 ± 6.2	51.9 ± 7.6	55.0 ± 6.5	49.8 ± 4.1	0.0 ± 0.0	0.0 ± 0.0
Grade B	27.6 ± 0.6	29.5 ± 2.0	32.6 ± 1.6	23.6 ± 2.1	26.8 ± 8.6	25.2 ± 5.6	10.0 ± 10.0	8.2 ± 1.9
A + B	79.9 ± 5.0[a]	88.3 ± 2.4[b]	80.1 ± 7.7[b]	75.5 ± 9.7[b]	81.7 ± 2.1[b]	75.0 ± 9.7[b]	10.0 ± 10.0[c]	8.2 ± 1.9[d]
Grade C	19.7 ± 4.5	12.2 ± 2.8	19.1 ± 6.9	23.9 ± 10.4	18.4 ± 2.2	22.7 ± 9.9	58.7 ± 11.3	27.5 ± 2.5
Grade D	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.7	0.0 ± 0.0	2.4 ± 0.2	31.3 ± 21.3	64.4 ± 4.4
C + D	20.1 ± 5.0	12.2 ± 2.8	19.1 ± 6.0	24.6 ± 9.7	18.4 ± 2.2	25.1 ± 9.7	90.0 ± 10.0	91.9 ± 1.9

Grade A colony indicates colony with >80% undifferentiated; grade B colony, colony with 50%–80% undifferentiated; grade C colony, colony with <50% undifferentiated; grade D colony, unattached, dead, or lysed colony.

[a,c; a,d; b,c; b,d] $p < .001$.

-Acceptable survival rate
-Quick and rapid and less toxicity
-Small quantity of hESC colonies (10-15)

Richards et al, 2004

Freezing methods

BIOTECHNOLOGY AND BIOENGINEERING, VOL. 88, NO. 3, NOVEMBER 5, 2004

Cryopreservation of Adherent Human Embryonic Stem Cells

Lin Ji, Juan J. de Pablo, Sean P. Palecek

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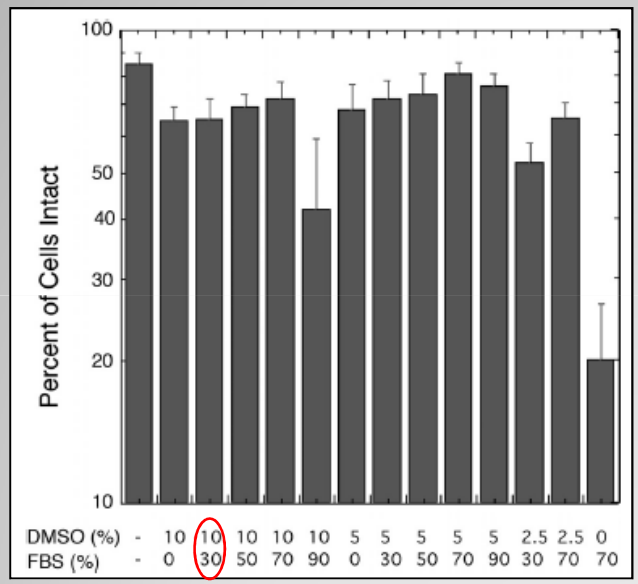
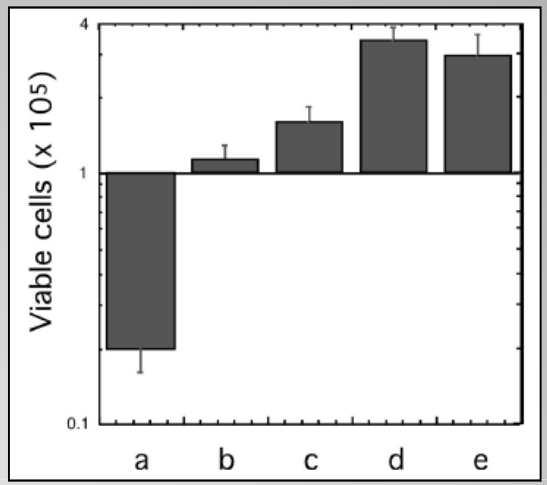
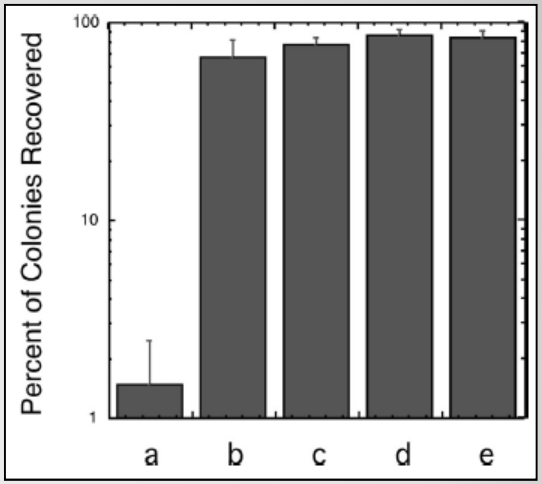
Published online 12 October 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20243

-Slow method (differents % of FBS, DMSO and CM) suspension vrs adherent way in matrigel and protected with Disaccharide trehalose
-H1 and H9
-Using programmable freezer

Freezing in adherent system







-Better survival rate
-Large quantity of hESC cells (106)
-No differentiation was studied

Ji et al, 2004

Freezing methods

Human Reproduction Vol.20, No.7 pp. 1779–1785, 2005
doi:10.1093/humrep/deh854
Advance Access publication March 10, 2005

Cryopreservation of human embryonic stem cells without the use of a programmable freezer

Sung Yun Ha[1], Byung Chul Jee[3], Chang Suk Suh[1,2,3,4], Hee Sun Kim[2], Sun Kyung Oh[2], Seok Hyun Kim[1,2] and Shin Yong Moon[1,2]

-Slow method without programmable freezer
-(FBS 95,50,5%), DMSO (5,10%), EG (5,10,20%) and grycerol (5,10,20%)
-SNUhES-3 line

Freezing methods

Table I. The survival rates of human embryonic stem (ES) cells after slow freezing-rapid thawing using various medium compositions

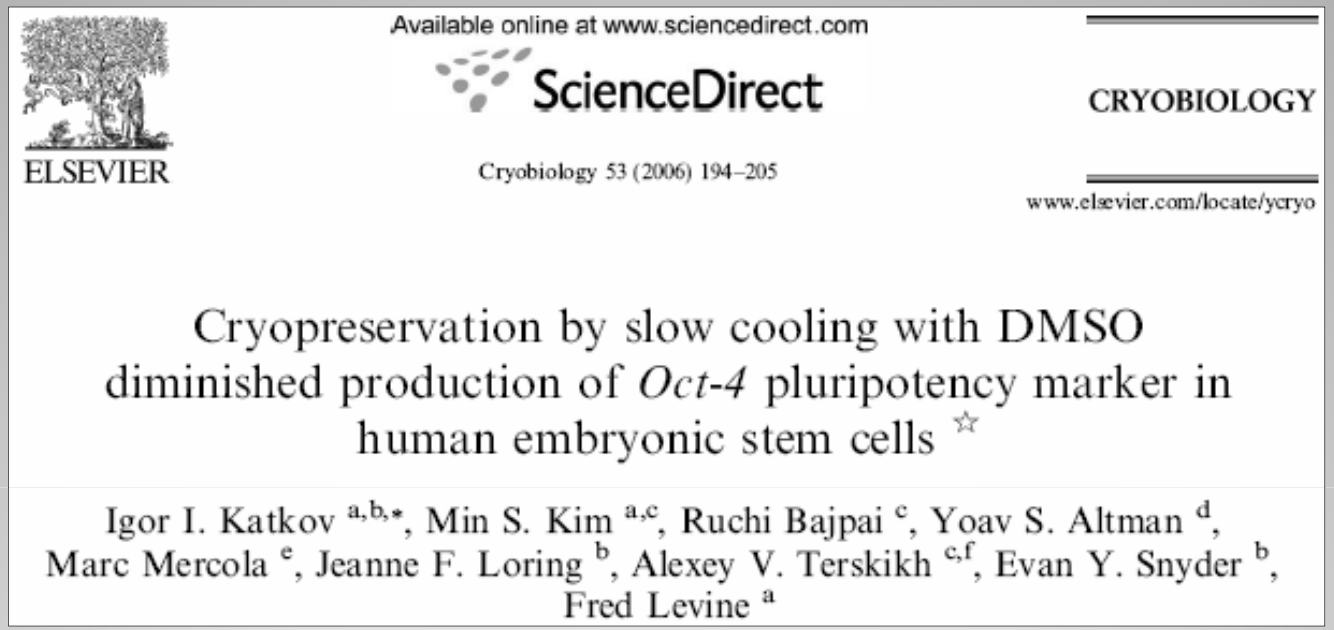
Experiments	Cryoprotectant compositions	Fetal bovine serum (%)	*n*	Survival rate (%)
I	5% DMSO	95	6	11.3 ± 6.5^{a}
	10% DMSO	90	6	1.3 ± 0.17^{b}
	5% EG	95	6	0.3 ± 0.3^{b}
	10% EG	90	6	0
	5% glycerol	95	6	0
II	5% DMSO	95	6	11.0 ± 6.9^{c}
	5% DMSO	50	6	9.3 ± 3.9^{d}
	5% DMSO	5	6	1.5 ± 0.1
III	5% DMSO + 5% EG	50	6	4.7 ± 1.5^{e}
	5% DMSO + 10% EG	50	6	30.2 ± 1.6^{f}
	5% DMSO + 20% EG	50	6	0
	5% DMSO + 5% glycerol	50	6	0
	5% DMSO + 10% glycerol	50	6	0
	5% DMSO + 20% glycerol	50	6	0

n = number of repeated experiments. Each experiment included 100 colonies of human ES cells.
Survival rates are represented as means ± SEM.
$^{a,b}P < 0.05$, ^{c,A}not significant, $^{d,f}P < 0.05$, $^{e,f}P < 0.05$ (Mann–Whitney *U*-test, two-tailed).
DMSO = dimethylsulphoxide; EG = ethylene glycol.

-Better survival rate
-No differentiation was studied
-Large quantity of hESC colonies (100)

Ha et al, 2005

Freezing methods



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Cryobiology 53 (2006) 194–205

CRYOBIOLOGY

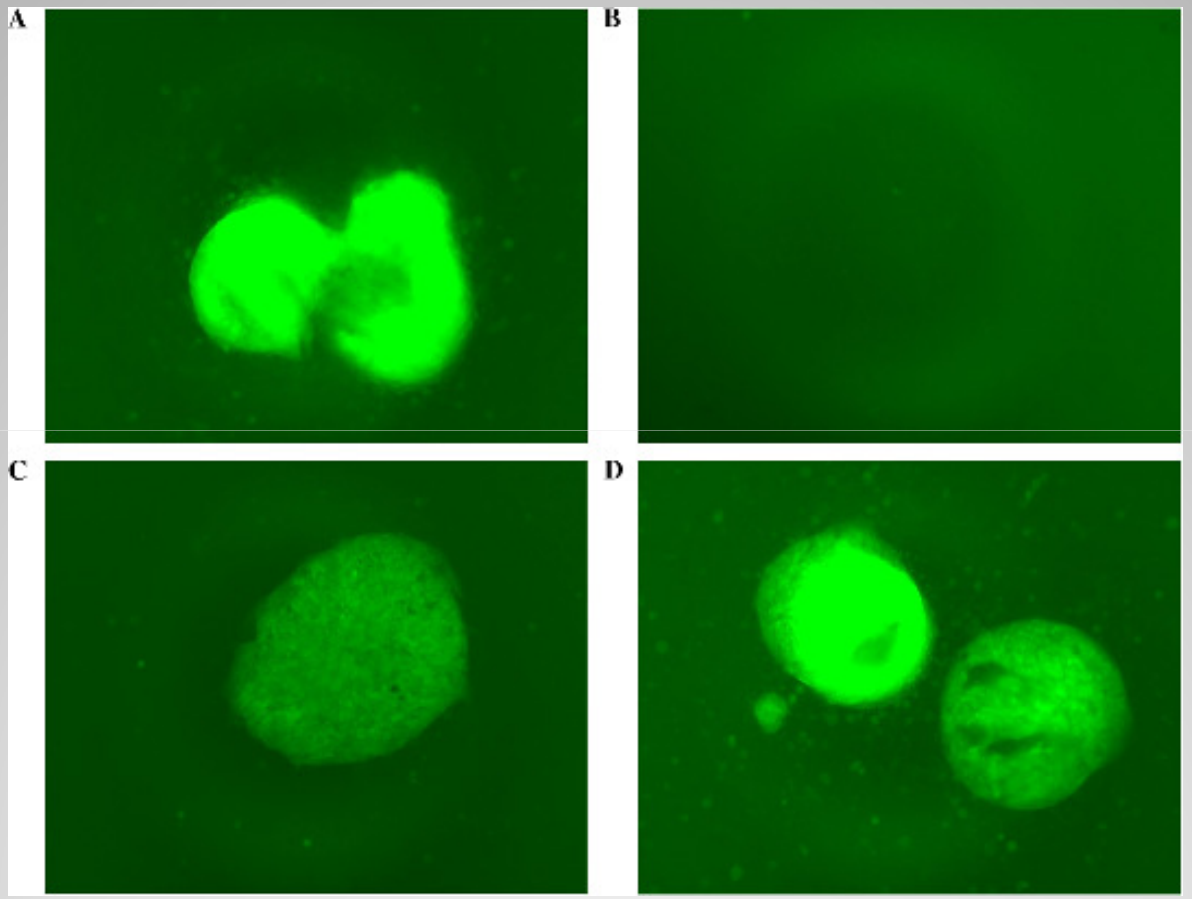
www.elsevier.com/locate/ycryo

Cryopreservation by slow cooling with DMSO diminished production of *Oct-4* pluripotency marker in human embryonic stem cells ☆

Igor I. Katkov [a,b,*], Min S. Kim [a,c], Ruchi Bajpai [c], Yoav S. Altman [d], Marc Mercola [e], Jeanne F. Loring [b], Alexey V. Terskikh [c,f], Evan Y. Snyder [b], Fred Levine [a]

- Slow cooling H9 DMSO (10%) WiCell protocol
- Short and long term storage
- Endogenous and Oct-4-EGFP reporter by FM and FCM
- Cell death (50% PI) and EGFP loosed (10%)

Freezing methods



Freezing methods

Biosci Rep (2007) 27:257–264
DOI 10.1007/s10540-007-9051-2

ORIGINAL PAPER

Caspase Inhibitor Z-VAD-FMK Enhances the Freeze-Thaw Survival Rate of Human Embryonic Stem Cells

Boon Chin Heng · Marie Veronique Clement · Tong Cao

-Cryopreservation induce apoptosis (Heng et al, 2006)

-Survival rate enhancement in H1

-WiCell protocol: DMEM/F12 medium, DMSO (10%) and FBS (20%):www.wicell.org/uploads/media/Freezing_HESC

Table 1 Exposure to 100 mM Z-VAD-FMK in the freezing solution did not significantly enhance the post-thaw survival rate ($p > 0.05$, Student's unpaired t-test). However, when 100 mM Z-VAD-FMK was added to the post-thaw culture media, there was a significant enhancement ($p < 0.05$, Student's unpaired t-test), which was further improved when Z-VAD-FMK was also added to the freezing solution ($p < 0.01$, Student's unpaired t-test)

	Raw absorbance values obtained for MTT assay (after correction for blank, $n = 5$)	% Post-thaw survival rate
Non-cryopreserved control	2.84 ± 0.06	–
Cryopreservation without Z-VAD-FMK	0.28 ± 0.01[a,b,c]	9.9%
Cryopreservation with 100 mM Z-VAD-FMK in freezing solution	0.29 ± 0.03[a]	10.2%
Cryopreservation with 100 mM Z-VAD-FMK in post-thaw culture media	0.41 ± 0.04[b]	14.4%
Cryopreservation with 100 mM Z-VAD-FMK in both freezing solution and post-thaw culture media	0.53 ± 0.05[c]	18.7%

The post-thaw survival rates were computed by dividing the MTT absorbance values obtained after cryopreservation with the absorbance reading for the non-cryopreserved control

[a] Not significantly different ($p > 0.05$)

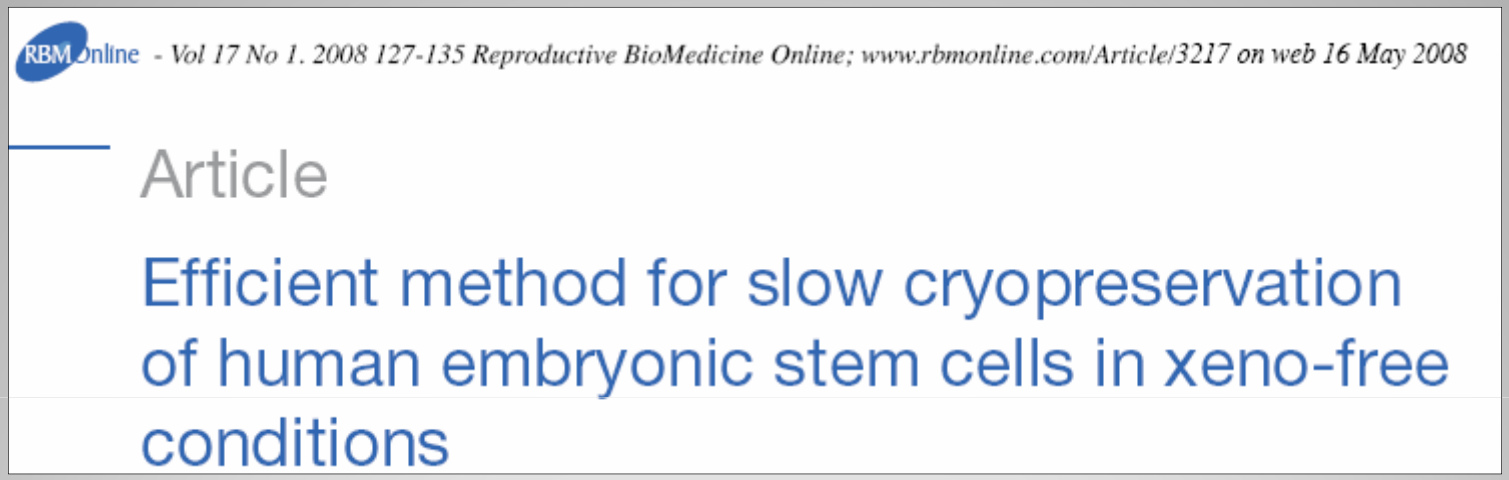
[b] Significantly different ($p < 0.05$)

[c] Significantly different ($p < 0.01$)

Heng et al, 2007

-Spontaneous differentiation was not inhibited

Freezing methods



-Slow method using DMSO
-Large quantity of hESC colonies (50-100)

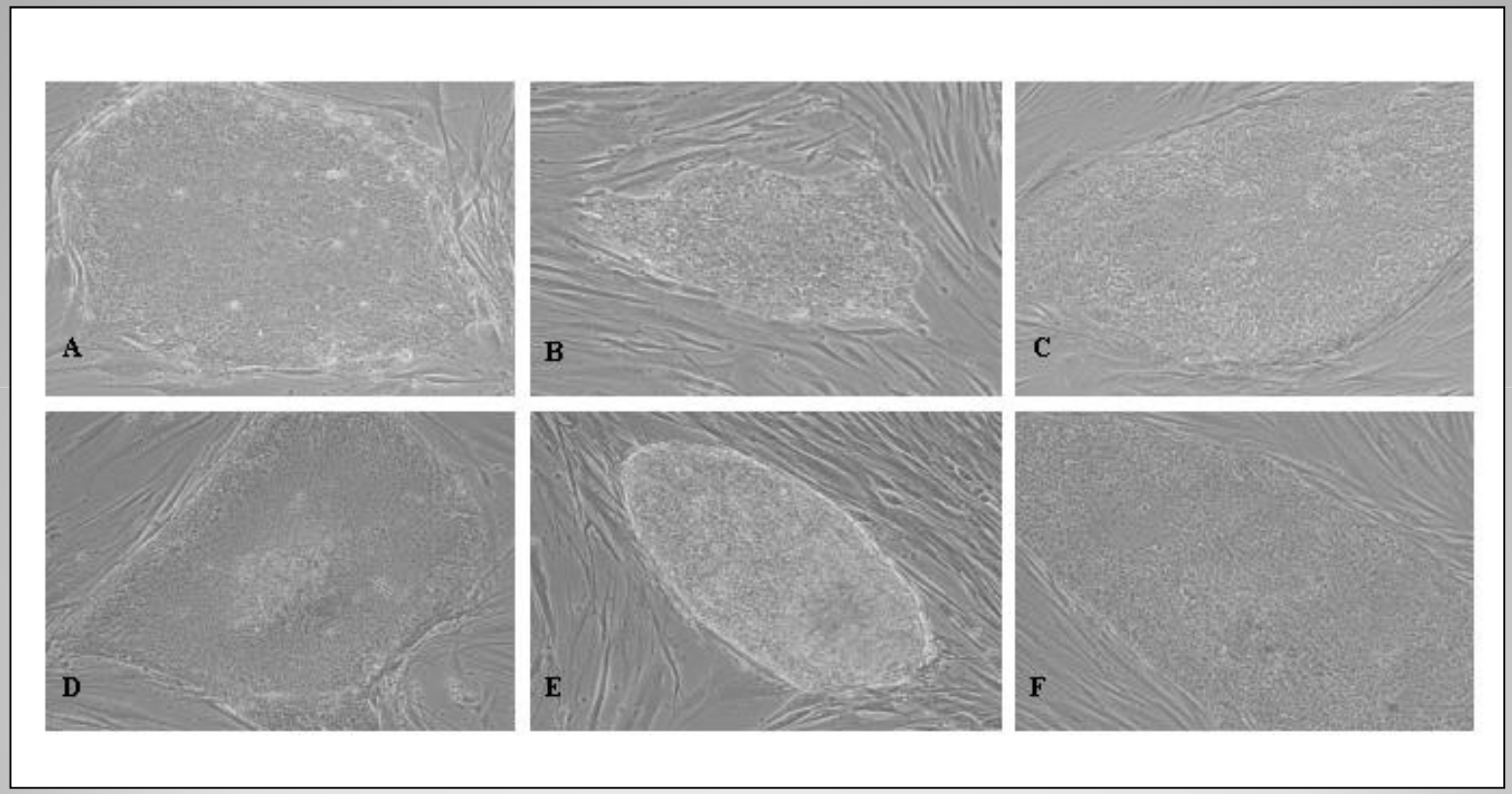
Valbuena et al, 2008

Survival rates

		Control 1 (Reubinoff *et al,* 2001)		Control 2 (Richards *et al,* 2004)		Valencia Method (with programmable freezer)		Valencia Method (without programmable Freezer)	
Survival rate %	VAL-3	*15,3±2,1*	A. 70,6±2,5	*2,2±0,4*	A. 83,7±3,0	*41,3±2,2*	A. 70,4±2,5	*68,0±3,4*	A. 84,3±1,6
			B. 19,4±1,6		B. 11,2±0,9		B. 19,6±1,2		B. 10,7±1,0
	VAL-5	*13,3±1,1*	A. 54,5±2,1	*2,2±0,5*	A. 38,5±1,9	*8,5±1,1*	A. 69,3±2,2	*15,1±2,8*	A. 59,6±2,3
			B. 44,9±1,8		B. 53,8±2,4		B. 15,3±0,2		B. 30,2±1,6
dA	VAL-3	4±1		14±1		7±0		6±0	
	VAL-5	13±1		20±2		8±0		6±0	
	VAL-3	6±0		16±1		11±1		10±1	

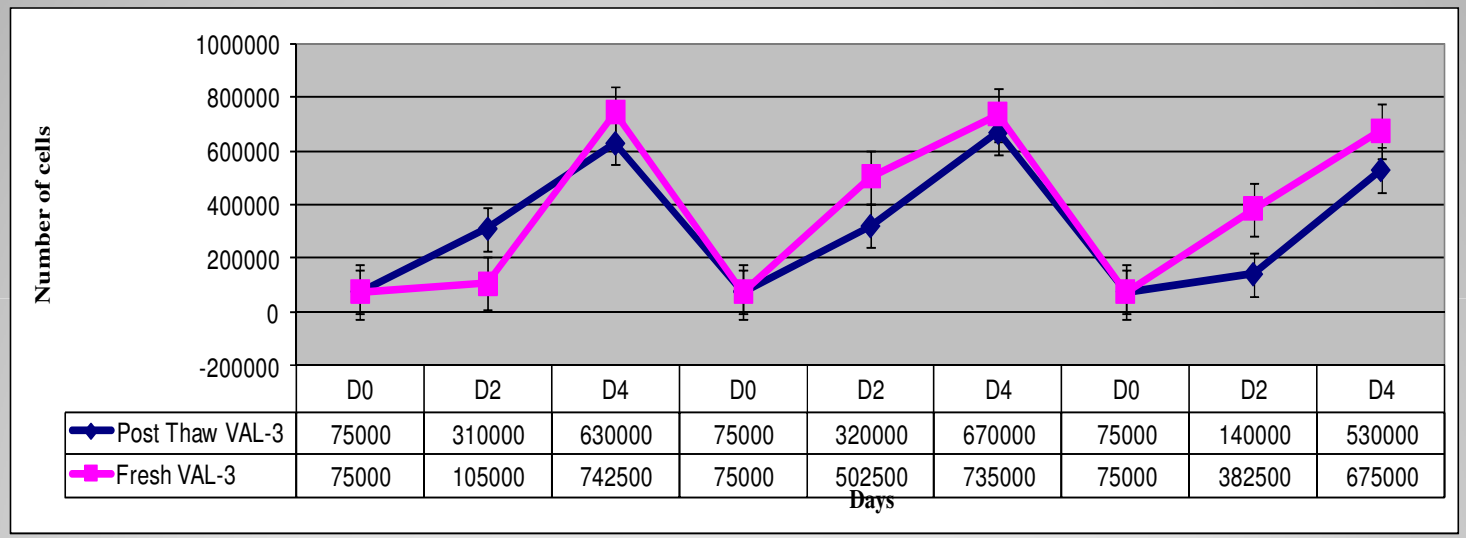
Valbuena et al, 2008

Morphology



Valbuena et al, 2008

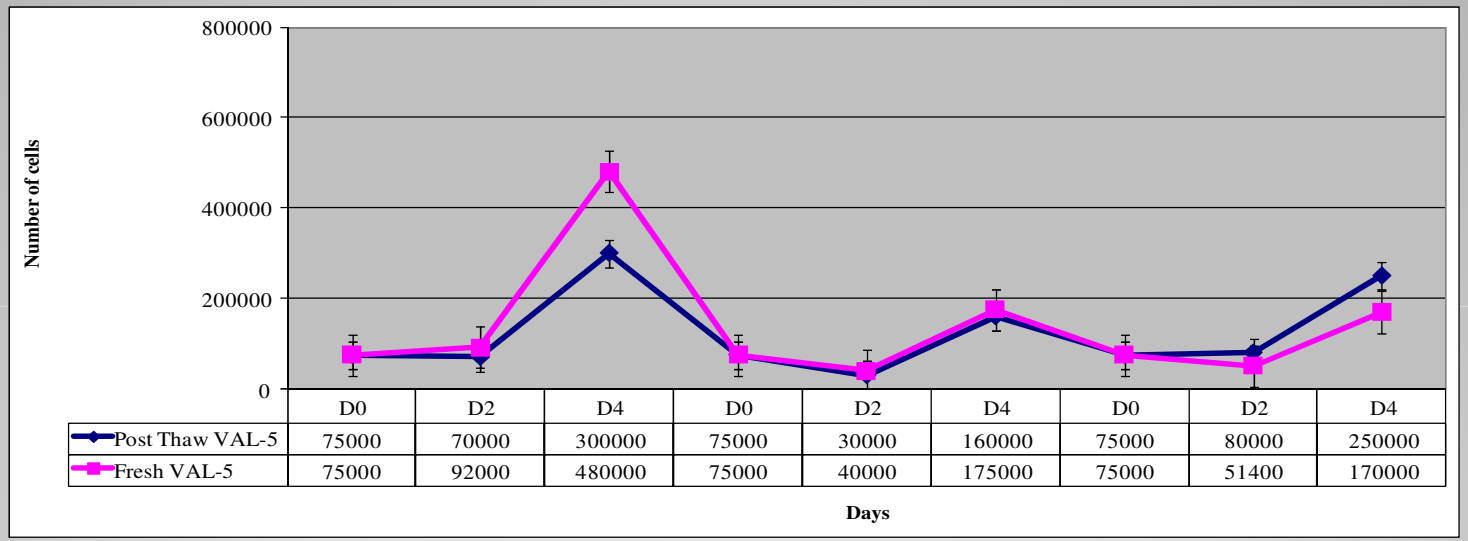
Growth curve of VAL-3



	D0	D2	D4	D0	D2	D4	D0	D2	D4
Post Thaw VAL-3	75000	310000	630000	75000	320000	670000	75000	140000	530000
Fresh VAL-3	75000	105000	742500	75000	502500	735000	75000	382500	675000

Valbuena et al, 2008

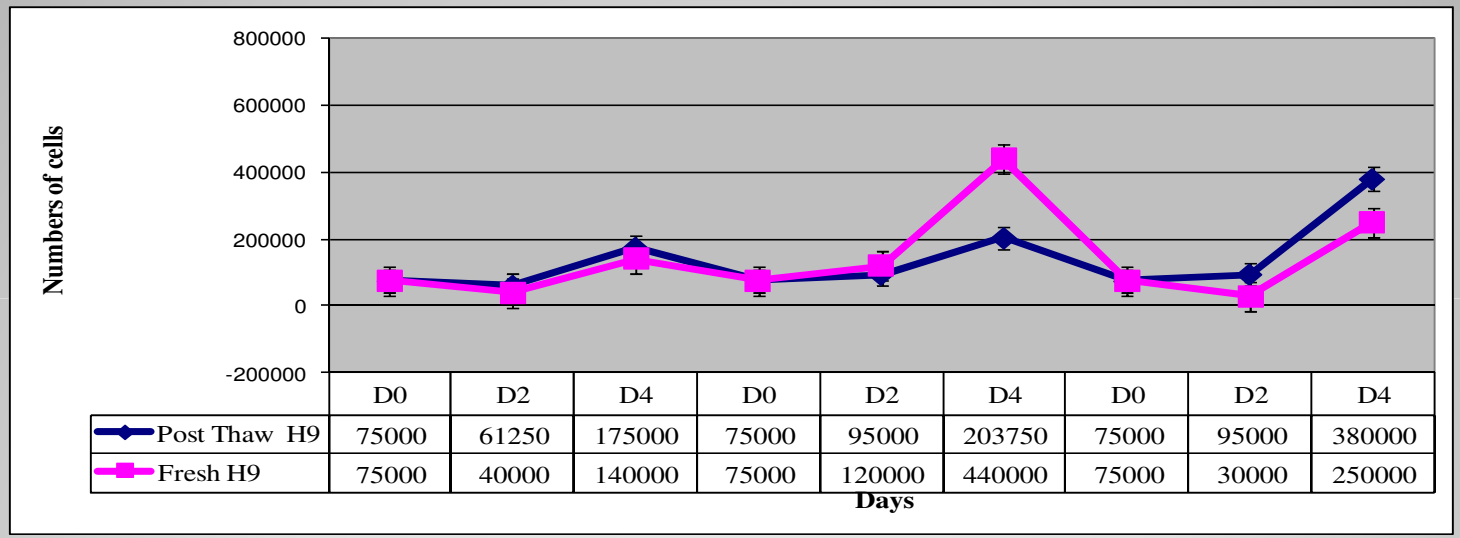
Growth curve of VAL-5



	D0	D2	D4	D0	D2	D4	D0	D2	D4
Post Thaw VAL-5	75000	70000	300000	75000	30000	160000	75000	80000	250000
Fresh VAL-5	75000	92000	480000	75000	40000	175000	75000	51400	170000

Valbuena et al, 2008

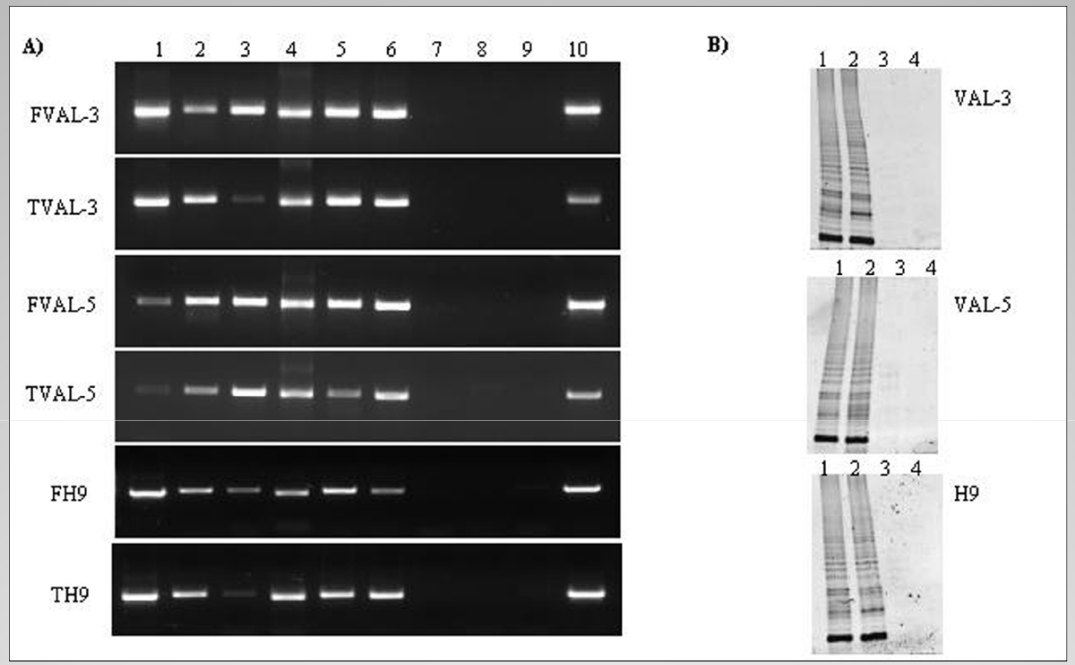
Growth curve of H9



	D0	D2	D4	D0	D2	D4	D0	D2	D4
Post Thaw H9	75000	61250	175000	75000	95000	203750	75000	95000	380000
Fresh H9	75000	40000	140000	75000	120000	440000	75000	30000	250000

Valbuena et al, 2008

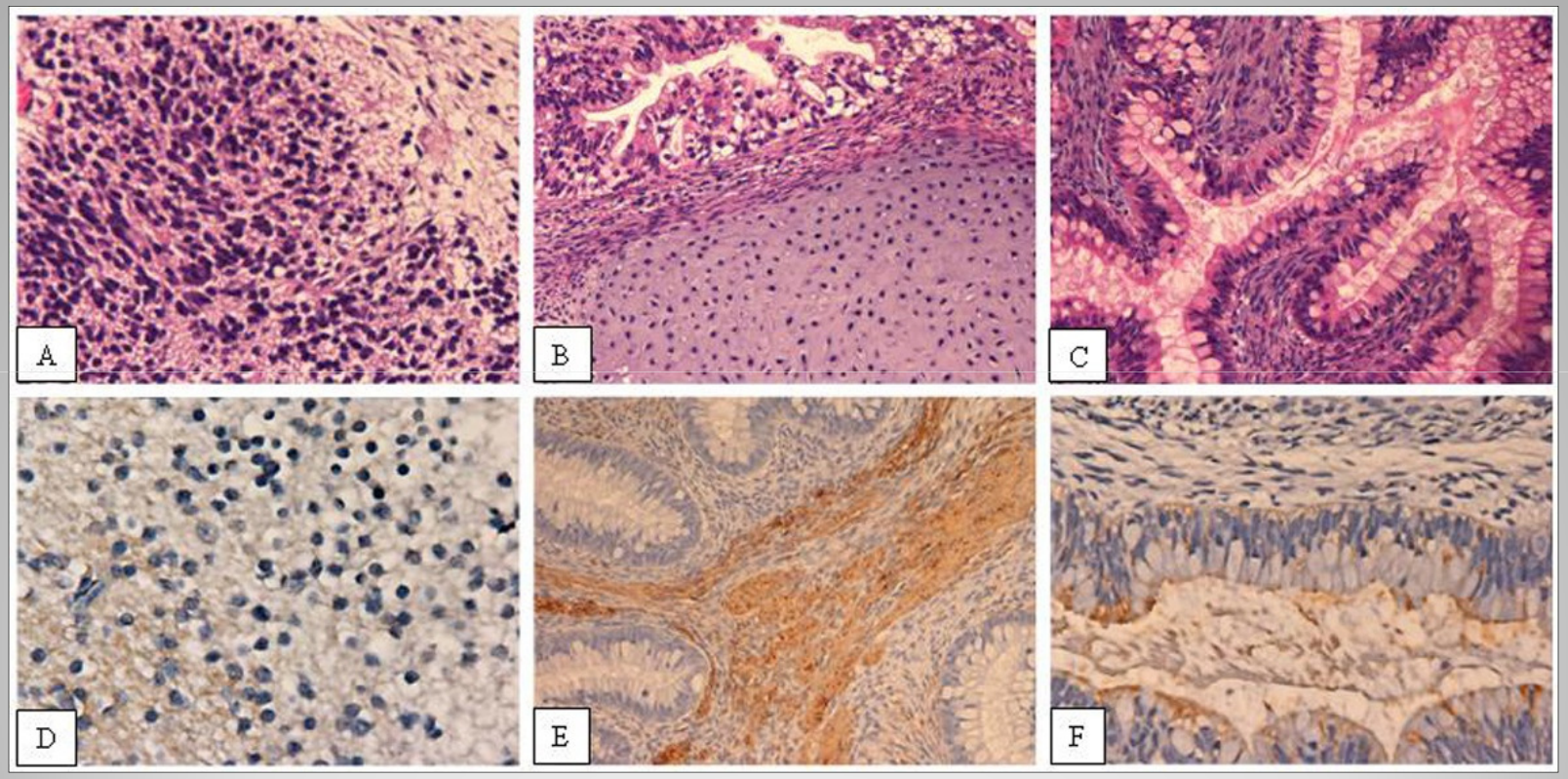
Undifferentiated stage



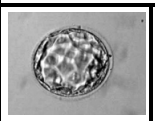
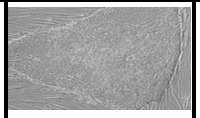
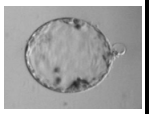
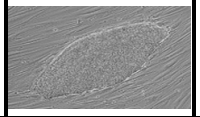
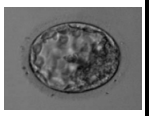
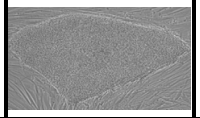
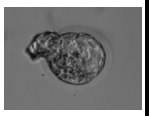
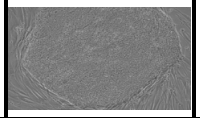
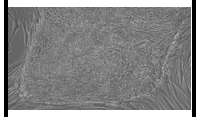
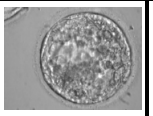
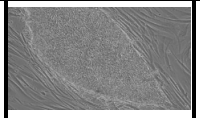
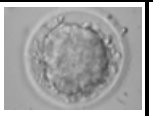
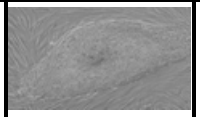
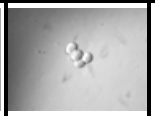
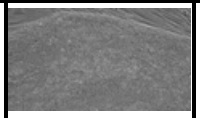
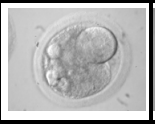
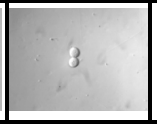
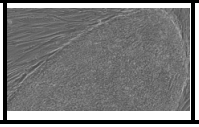
1.Oct-3/4
2.Sox-2
3.Rex-1
4.Nanog
5. Cripto
6.Thy-1
7.Ectoderm, Nfh
8.Mesoderm, Ren
9.Endoderm, amylase
10.GAPDH

Valbuena et al, 2008

Posthaw H9 in vivo Differentiation



Valbuena et al, 2008

Cell Line	Embryo		Colony Morphology		Last passage Criovials
VAL-3		Whole normal blastocyst stage embryo (46,XY) derivation in human foreskin		10X, P42	80 29 CV
VAL-4		Whole normal blastocyst stage embryo (46,XX) derivation in human foreskin		10X, P40	54 38 CV
VAL-5		Whole normal blastocyst stage embryo (46,XX) derivation in human foreskin		10X, P43	49 37 CV
VAL-6M		Whole monogenetic affected embryo derivation (DM Type1) (46,XY) in human foreskin		10X, P45	49 29 CV
VAL-7		Derivation from ICM isolated by laser (46,XY) in human foreskin		10X, P22	65 18 CV
VAL-8		Whole normal blastocyst stage embryo (46,XX) derivation in human foreskin		10X, P6	22 30 CV
VAL-9		Whole normal morula stage embryo (46,XY) derivation in human foreskin		10X, P6	55 43 CV
VAL-10B		 Derivation from single biopsied blastomere (46,XY) in human foreskin		10x, P13	25 39 CV
VAL-11B		 Derivation from single biopsied blastomere (46,XX) in human foreskin		10x, P15	25 46 CV

Survival rate per colony

hESC Line	Cryopreserved colonies (n/ vial)	Day of first passage	Successfully thawed colonies (n/vial)	Survival rate per cryopreserved colony (%)	Survival rate per cryovial (%)
VAL-3	55,6	7,9	8,9	15,7	94,4
VAL-4	57,6	11,1	16,5	18,2	60
VAL-5	53,5	9	3,9	6	43,5
VAL-6	30	10	7	23,3	80
VAL-7	35	5	5	14,29	50
VAL-8	30	7	4,7	15,6	33,3
VAL-9	30	7,5	12	40	80
VAL-10B	30	9,7	23,3	77,8	100
VAL-11B	33	9,8	10	30,5	100
H9	58,0	6,8	16,1	28,8	75
Global results	**41,3**	**8,4**	**10,74**	**27,0 (%)**	**71,62 (%)**

Valbuena et al, Cryomeeting 2010

Freezing methods

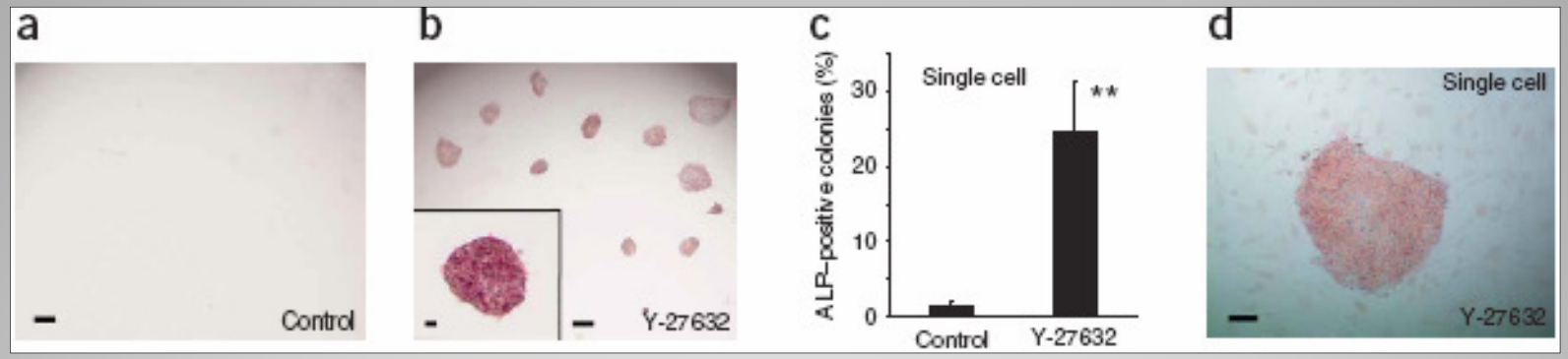
nature biotechnology

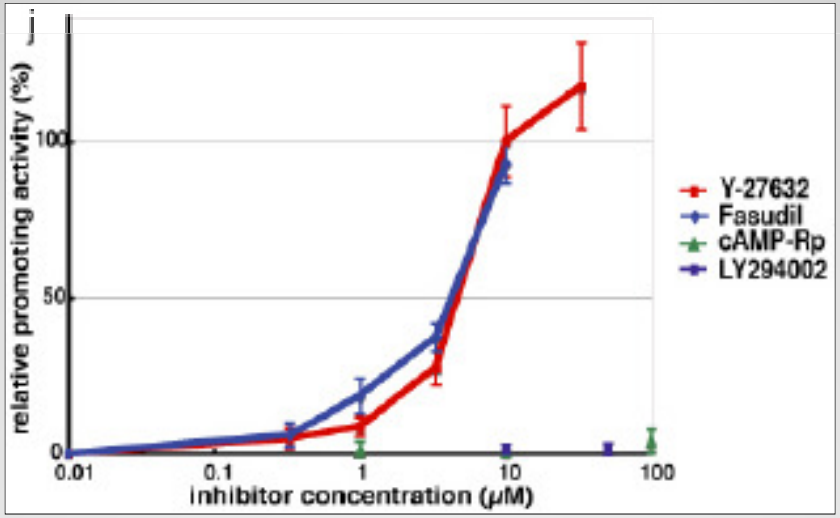
A ROCK inhibitor permits survival of dissociated human embryonic stem cells

Kiichi Watanabe[1,5], Morio Ueno[1,3], Daisuke Kamiya[1], Ayaka Nishiyama[1], Michiru Matsumura[1], Takafumi Wataya[1,4], Jun B Takahashi[4], Satomi Nishikawa[2], Shin-ichi Nishikawa[2], Keiko Muguruma[1] & Yoshiki Sasai[1]

- Cryopreservation induce apoptosis (Heng et al, 2006)
- KhES-1, 2 and 3 lines

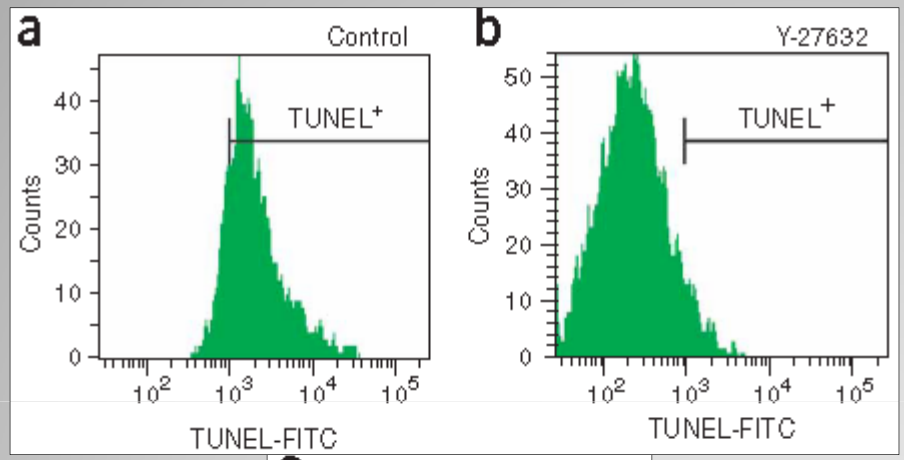
Freezing methods

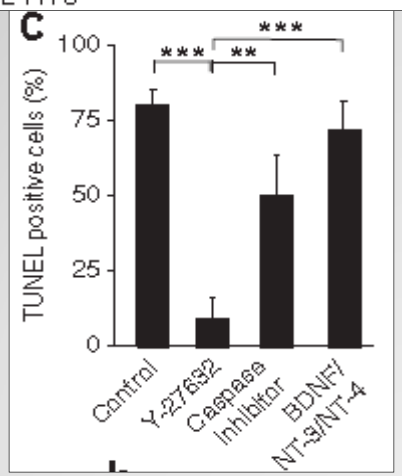




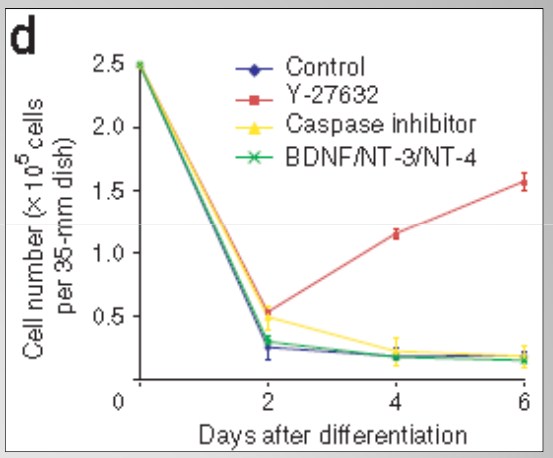
Watanabe et al, 2007

Freezing methods





Apoptosis



Survival rate

Watanabe et al, 2007

Freezing methods

STEM CELLS AND DEVELOPMENT 17:1079–1086 (2008)

DOI: 10.1089/scd.2007.0247

The ROCK Inhibitor Y-27632 Enhances the Survival Rate of Human Embryonic Stem Cells Following Cryopreservation

Xiangyun Li,[1,2,*] Guoliang Meng,[1,*] Roman Krawetz,[1] Shiying Liu,[1] and Derrick E. Rancourt[1]

-Slow freezing (90% KSR+10%DMSO)

-CA1,CA2,H9,H1 lines

Freezing methods

TABLE 1. SURVIVAL AND DIFFERENTIATION RATES OF hES CELLS AFTER SLOW FREEZING AND RAPID THAWING

Cell lines	Groups	Survival rate (%) at day 5 after thawing[a]	Differentiation rate (%) at different passages after thawing[b]			
			I	II	III	IV
CA1	Fresh control[c]	283/302 (93.7)	14/215 (6.5)	16/173 (9.2)	12/150 (8.0)	19/186 (10.2)
	Frozen control[d]	38/456 (8.3)[g]	11/210 (5.2)	96/324 (29.6)[g]	18/247 (7.3)	26/270 (9.6)
	Y-27632 group[e]	422/485 (87.0)[f,g]	30/350 (8.6)	122/362 (33.7)[g]	37/335 (11.0)	17/294 (5.8)
H9	Fresh control[c]	178/185 (96.2)	18/158 (11.4)	22/203 (10.8)	11/127 (8.7)	9/96 (9.4)
	Frozen control[d]	49/320 (15.3)[g]	38/270 (14.1)	40/167 (24.0)[g]	9/85 (10.6)	20/150 (13.3)
	Y-27632 group[e]	225/246 (91.5)[f,g]	35/220 (15.9)	73/282 (25.9)[g]	24/215 (11.2)	20/162 (12.3)

[a]Number of colonies on day 5/total colonies replated.
[b]Number of differentiated colonies/total colonies replated.
[c]Cells without freezing.
[d]Cells without Y-27632 treatment frozen by the slow-freezing protocols.
[e]Cells with Y-27632 treatment frozen by the slow-freezing protocols.
[f]$p < 0.01$, frozen control group versus Y-27632 treatment group in the same cell line.
[g]$p < 0.01$, fresh control group versus frozen control and Y-27632 treatment group in the same cell line.

-Better survival rate (87-91%)
-Does not inhibit spontaneous differentiation

Li et al, 2008

Freezing methods

Human Reproduction, Vol.24, No.3 pp. 580–589, 2009
Advanced Access publication on December 4, 2008 doi:10.1093/humrep/den404

human reproduction

ORIGINAL ARTICLE *Embryology*

ROCK inhibitor improves survival of cryopreserved serum/feeder-free single human embryonic stem cells

Xiangyun Li[1,2], Roman Krawetz[1], Shiying Liu[1], Guoliang Meng[1], and Derrick E. Rancourt[1,3]

-90% KSR, 10 DMSO
-Y-27632 in culture media before freezing and post-thaw
-ROCK inhibitor increases adherent properties and protects single apoptosis

Freezing methods

Human Reproduction, Vol.24, No.10 pp. 2468–2476, 2009
Advanced Access publication on July 14, 2009 doi:10.1093/humrep/dep244

human reproduction

ORIGINAL ARTICLE *Embryology*

A simple and efficient cryopreservation method for feeder-free dissociated human induced pluripotent stem cells and human embryonic stem cells

Sepideh Mollamohammadi[1], Adeleh Taei[1], Mohammad Pakzad[1], Mehdi Totonchi[1,2], Ali Seifinejad[1], Najmehsadat Masoudi[2], and Hossein Baharvand[1,3,4]

-Single dissociated hESC and iPS cells
-Y-27632 in freezing media vrs. freezing and post-thaw media

Freezing methods

Cryobiology 60 (2010) 344–350

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

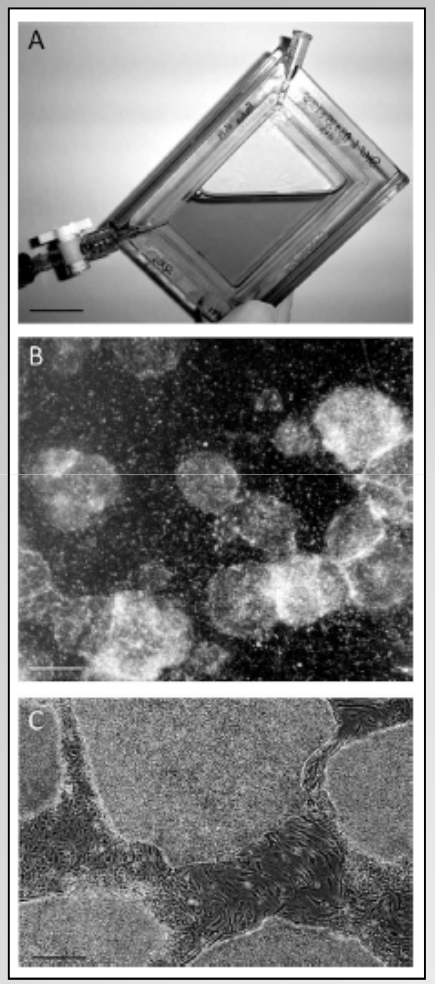
In situ cryopreservation of human embryonic stem cells in gas-permeable membrane culture cassettes for high post-thaw yield and good manufacturing practice ☆

K.J. Amps *, M. Jones, D. Baker, H.D. Moore

Centre for Stem Cell Biology, Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, UK

-FCS and MEF

Freezing methods



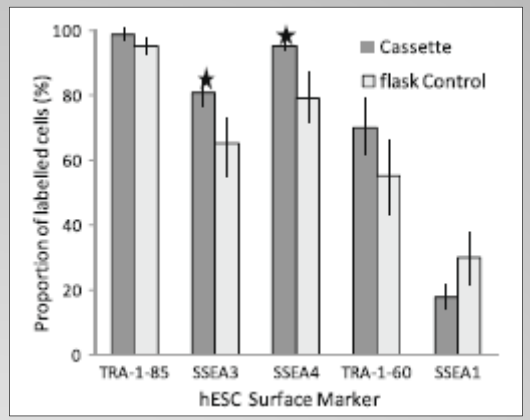


Table 1
Post-thaw proliferation rates for Shef hESC lines after cryovial or cassette freezing.

ShefhESC line (passage #)	Post-thaw cell proliferation (10^6 cells)		Proliferation ratio
	Cryovial/flask	Cassette	
4 (90)	3.2± 1.6	624 ±82**	195
5 (30)	1.2± 0.3	21.8 ±3.6*	17.5
6 (63)	2.4± 0.9	265 ±31**	115
7 (37)	1.5± 0.4	12.4 ±2.4*	8.2

* Significantly different $P \leq 0.05$.
** Significantly different $P \leq 0.001$. $n = 3$ for each treatment.

-Undifferentiation stage better in cassette than cryovials
-High proliferation rates

Conclusions

- The state of the art of hESC cryopreservation has been presented.
- We have developed an alternative freezing method without serum (Xeno-free).
- Survival rates are similar to stablished methods maintaining undifferentiation potential.
- Rock inhibitor is a promissing tool to improve cryopreservation efficiency.