In Vitro Cell.Dev.Biol.—Animal (2010) 46:247–258
DOI 10.1007/s11626-010-9297-z

Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells

The International Stem Cell Initiative Consortium · Veronika Akopian · Peter W. Andrews · Stephen Beil · Nissim Benvenisty · Jennifer Brehm · Megan Christie · Angela Ford · Victoria Fox · Paul J. Gokhale · Lyn Healy · Frida Holm · Outi Hovatta · Barbara B. Knowles · Tenneille E. Ludwig · Ronald D. G. McKay · Takamichi Miyazaki · Norio Nakatsuji · Steve K. W. Oh · Martin F. Pera · Janet Rossant · Glyn N. Stacey · Hirofumi Suemori

Received: 11 January 2010 / Accepted: 15 January 2010 / Published online: 26 February 2010 / Editor: P. Andrews

Abstract There are many reports of defined culture systems for the propagation of human embryonic stem cells in the absence of feeder cell support, but no previous study has undertaken a multi-laboratory comparison of these diverse methodologies. In this study, five separate laboratories, each with experience in human embryonic stem cell culture, used a panel of ten embryonic stem cell lines (including WA09 as an index cell line common to all

V. Akopian · S. Beil · V. Fox · M. F. Pera
The Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research at USC, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA

P. W. Andrews (✉) · A. Ford · P. J. Gokhale
Centre for Stem Cell Biology, Department of Biomedical Science, The University of Sheffield, Sheffield S10 2TN, UK
e-mail: p.w.andrews@sheffield.ac.uk

P. J. Gokhale
e-mail: p.j.gokhale@sheffield.ac.uk

N. Benvenisty
Institute of Life Sciences, Department of Genetics, The Hebrew University of Jerusalem, Edmond J. Safra Campus, Givat Ram, Jerusalem 91904, Israel

J. Brehm · T. E. Ludwig
WiCell Research Institute, P.O. Box 7365, Madison, WI 53707-7365, USA

M. Christie · L. Healy · G. N. Stacey
UK Stem Cell Bank, Division of Cell Biology and Imaging, National Institute for Biological Standards and Control-Health Protection Agency, South Mimms, Herts EN6 3QG, UK

F. Holm · O. Hovatta
Karolinska Institutet, Department of Clinical Science, Technology and Intervention, Karolinska University Hospital, SE 141 86 Stockholm, Sweden

B. B. Knowles
Institute of Medical Biology 8A Biomedical Grove, #06-06 Immunos, Singapore 138648, Republic of Singapore

R. D. G. McKay
NIH Stem Cell Unit, National Institutes of Health, Bldg 35/2B-213, MSC 3703, 9000 Rockville Pike, Bethesda, MD 20892, USA

T. Miyazaki · N. Nakatsuji · H. Suemori
Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

S. K. W. Oh
Bioprocessing Technology Institute, Agency for Science Technology and Research (A*STAR), 20 Biopolis Way, #06-01 Centros, Singapore 138668, Republic of Singapore

J. Rossant
Developmental Biology Program, The Hospital for Sick Children, TMDT Building, Room 13-305, 101 College Street, Toronto, ON M5G 1L7, Canada

laboratories) to assess eight cell culture methods, with propagation in the presence of Knockout Serum Replacer, FGF-2, and mouse embryonic fibroblast feeder cell layers serving as a positive control. The cultures were assessed for up to ten passages for attachment, death, and differentiated morphology by phase contrast microscopy, for growth by serial cell counts, and for maintenance of stem cell surface marker expression by flow cytometry. Of the eight culture systems, only the control and those based on two commercial media, mTeSR1 and STEMPRO, supported maintenance of most cell lines for ten passages. Cultures grown in the remaining media failed before this point due to lack of attachment, cell death, or overt cell differentiation. Possible explanations for relative success of the commercial formulations in this study, and the lack of success with other formulations from academic groups compared to previously published results, include: the complex combination of growth factors present in the commercial preparations; improved development, manufacture, and quality control in the commercial products; differences in epigenetic adaptation to culture in vitro between different ES cell lines grown in different laboratories.

Keywords Human embryonic stem cell · Cell culture · Defined cell culture media · Comparative study

Introduction

The potential for the use of human embryonic stem cells (hESC) and human-induced pluripotent stem (hiPS) cells in research and therapy is widely recognized, but progress in the field depends critically on well-characterized systems for stem cell growth and differentiation. The original culture systems for the derivation and maintenance of hESC employed basal medium supplemented with fetal calf serum and mouse embryonic fibroblast feeder cell layer support (Thomson et al. 1998; Reubinoff et al. 2000). The presence of undefined components of animal origin in these systems is problematic, for two main reasons. First, unknown factors present in serum or produced by the feeder cell layers may confound interpretation of studies of the effect of exogenous agents on the growth and differentiation of the stem cells. Second, components such as serum, growth factors, and feeder cells are prone to significant variability and better defined and more reproducible media will enhance standardization and help to minimize variation in stem cell cultures. It is also desirable to eliminate animal products as they may have the potential for transmission of pathogens to the cultured cells, presenting a barrier to future clinical application of hESC derivatives in therapy.

For these reasons, many research groups have set about developing more standardized and defined media formulations, sometimes based on the analysis of signaling systems required for hES self-renewal [reviews see (Chase and Firpo 2007; Unger et al. 2008)]. While most of the these studies have carefully validated the new formulations for the ability to support long-term maintenance of hESC, in general the reports focus on one or two cell lines often grown in the laboratory of origin. It remains unclear how robust are the different formulations, whether some perform better than others, whether a particular formulation will support a wide variety of cell lines, and how easy it is to transfer the published protocols between laboratories. There is a strong rationale for identifying a few common tissue culture platforms for hESC and hiPS cells, to enable development of standardized protocols for stem cell growth and differentiation, and to facilitate comparisons of studies between cell lines and between laboratories.

The International Stem Cell Initiative is a consortium of laboratories founded to help establish standards for pluripotent stem cell research (Andrews et al. 2005). Following a major survey of the phenotype of a large number of hESC isolates (Adewumi et al. 2007), the consortium decided to undertake a comparative study of defined culture systems for hESC growth. Participant laboratories were polled to identify a short list of the most promising formulations for study. The media chosen for study range from relatively simple formulations such as hESF9 which consists of a basal medium and FGF-2 supplemented with heparin sulfate (Furue et al. 2008), through complex media such as mTeSR1 (Ludwig et al. 2006a, b) and STEMPRO (Wang et al. 2007) which utilize several growth factors or chemicals which can mimic growth factor signaling to promote hES cell growth. The media often contain additives that serve as substitutes for serum-derived components, for example transferrin, albumin, cholesterol, and lipid mixtures. While all media we tested contained growth factors, there was a high degree of variation in the growth factors and concentrations used. For example, fibroblast growth factor was a component in all media but at a range of concentrations. The two most complex media in terms of growth factors and signaling agonists added were the commercial media mTeSR1 (Ludwig et al. 2006b) and STEMPRO (Wang et al. 2007). Interestingly, while both these media use stimulation of the FGF and TGF-beta pathway via FGF-2 and TGF-beta in mTeSR1 and FGF and ActivinA in STEMPRO, they also utilize alternate signaling pathways in addition to these two. mTeSR1 uses a GABA agonist and the non-specific WNT antagonist lithium chloride (Klein and Melton 1996), while STEMPRO uses the ErbB2 ligand HRG1beta and the insulin growth factor ligand LR3-IGF1.

This study addressed the ability of these diverse formulations to support hESC growth in academic laboratories experienced in hESC culture technology.

Table 1. Summary of laboratories and cell lines used in the study

Lab	CODE	Cell lines			
Kyoto University	KYOU	WA09 (H9)	KhES-1	KhES-3	
Karolinska Institute (KI)	KLNI	WA09 (H9)	HS181	HS420	
WiCell	WCEL	WA09 (H9)	WA13 (H13)	ES03 (HES3)	WA01 (H1)
CSCRM, University of Southern California	KUSC	WA09 (H9)	ES03 (HES3)	ES04 (HES4)	
UK Stem Cell Bank	UKSCB	WA09 (H9)	HUES9	NCL5	Shef2

Materials and Methods

Study Design. A meta-analysis of published methods including patent literature was conducted to identify likely candidate media. From this analysis, eight different culture systems (Li et al. 2005; Vallier et al. 2005; Liu et al. 2006; Lu et al. 2006; Ludwig et al. 2006a, b; Yao et al. 2006; Wang et al. 2007) were selected for further study by consortium laboratories. The media were designed to replace any feeder cell requirement, and extracellular matrix components were included as indicated by the laboratory of origin.

Four laboratories were recruited to conduct the initial study (see Table 1). These laboratories were asked to conduct tests using two cell lines from their own laboratory and also a centrally supplied reference cell line provided by WiCell, WA09 (Thomson et al. 1998). Cultivation of cells in the presence of Knockout Serum Replacer supplemented with FGF-2 in the presence of a mouse embryonic fibroblast feeder cell layer (Amit and Itskovitz-Eldor 2006) served as a positive control. Key components for the selected culture systems (such as growth factors and specialized additives) were purchased centrally and shipped to participating laboratories. The formulated media (or supplied commercial products) were tested in standardized cell attachment, survival, and maintenance (five to ten passages) assays using a small number of selected lines in the 4 central laboratories. Each culture in defined medium was initiated by seeding cells from stock cultures maintained under standard conditions used the test laboratory—generally culture on feeder cells in KSR-based media—with no allowance made to adapt cells to each of the new test media prior to the study. At each passage, the cells were seeded into multiple wells of a 6-well tissue culture plate, allowing replicate analyses as required during the trial. Protocols for cell culture, prepared by reference to the original published description of the defined media, and flow cytometry, were distributed to all participant groups. Details of these protocols may be found on the ISCI website (www.stemcellorg.com).

Once the original tests were completed, an independent laboratory (UK Stem Cell Bank, NIBSC-HPA) repeated studies on medias 1–6, using the original growth factor reagents, or newly purchased and formulated reagents, and in one case, fully supplemented medium ready to use,

Table 2. Summary of media and passaging regimes used in this study

Media no.	Media name	Reference	Passaging enzyme	Matrix	Notes
1		(Li et al. 2005)	Dispase	Geltrex or Matrigel	
2		(Liu et al. 2006)	Dispase	Geltrex or Matrigel	
3		(Vallier et al. 2005)	Dispase	Gelatin, MEF CM, 10% FBS	Use in 5% CO2 only. Passage when colonies 4–6 times size passaged from MEF-based cultures. Fibronectin can be used as alternative to FBS.
4		(Lu et al. 2006)	Dispase	Geltrex or Matrigel	
5		(Yao et al. 2006)	Dispase	Geltrex or Matrigel	
6	hESF9	(Furue et al. 2008)	EDTA, 0.2%	Collagen IA (Nitta Gelatin[a])	Passage day 2 after first passage, thence every 5 d. EDTA/Collagenase can used as alternative passaging reagents.
7	mTeSR1	(Ludwig et al. 2006a)	Dispase	Geltrex or Matrigel	Passage when colonies begin to merge together
8	STEMPRO	(Wang et al. 2007)	Dispase	Geltrex or Matrigel	Harvest cells 1–2 d after colonies touch. Maintain at >200 colonies/60 mm dish

[a] Nitta Geletin: Type I Collagen (Cellmatrix, Cell Science & Technology Institute, Inc. Japan)

supplied directly from the laboratory of origin of the formulation. The repeated testing was carried out on, with the independent cell lines HUES9, NCL5 and Shef2, and the H9 cell line common to all the laboratories (Table 1). The passaging regimes used in the study for each medium are summarized in Table 2.

Media Formulations. The formulations of the various test media, based on the published descriptions, are summarized in Table 3. A summary of the sources of the components that were used to formulate the different growth media is listed in Table 4.

Cell Growth. At each passage, three replicate wells of a 6-well plate were harvested with trypsin/EDTA, the cells were resuspended in PBSA containing trypan blue dye, and a viable cell count was performed.

Flow Cytometry. Flow cytometry analysis was carried out for nine cell surface antigens including eight markers of primate pluripotent stem cells and one differentiation marker as described elsewhere (Adewumi et al. 2007). 2102Ep human embryonal carcinoma cells were used as a positive control (Andrews et al. 1982; Josephson et al. 2007).

Table 3. Summary of media formulations used in the study

Media no.	1	2	3	4	5	6	7	8
KO-DMEM								
XVIVO-10	✓							
DMEM/F12		✓		✓	✓		✓[b]	✓
IMDM/F12			✓					
ESF[a]						✓		
N2		✓			✓			
B27		✓			✓			
NEAA	1%	1%			✓		✓	1×
L-glutamine	2 mM	1 mM	4 mM	1 mM	2 mM		2.94 mM	542 mg/l
Beta-MercaptoEthanol	0.1 mM	0.1 mM			0.11 mM	10 µM	0.098 mM	0.1 mM
Insulin			7 µg/ml	160 µg/ml		10 µg/ml	3.92 µM	
Transferrin			15 µg/ml	88 µg/ml		5 µg/ml	0.137 µM	10 µg/ml
2-ethanolamine						10 µM		
Na-selenite						20 nM		
L-ascorbic acid 2-phosphate						0.1 mg/ml		
Monothioglycerol			450 µM					
Cholesterol				✓			1.12 µM	
Lipids			✓			Oleic acid[c]	✓	Lipoic acid 0.105 mg/l
BSA			5 mg/ml		0.5 mg/ml	✓	0.195 mM	2 %
Pipecolic Acid							0.984 µM	
Activin A			10 ng/ml					10 ng/ml
bFGF	40 ng/ml	100 ng/ml	12 ng/ml	4 ng/ml	20 ng/ml	10 ng/ml	100 ng/ml	8 ng/ml
WNT3A				100 ng/ml				
hFLT3	15 ng/ml							
HRG1β								10 ng/ml
LR3-IGF1								200 ng/ml
BAFF				100 ng/ml				
TGF-beta							23.5 pM	
GABA							0.979 mM	
LiCl							0.98 mM	
Na Heparin SO4						✓		

[a] Base medium ESF designed for use with mouse ES cells (Furue et al. 2008)

[b] Modified DMEM/F12 (Ludwig et al. 2006b)

[c] Conjugated with fatty acid free BSA (9.4 µg/ml)

Table 4. Summary of sources of media components

Media no.	Media name	Reference	Component	Manufacturer	Catologue no.
1		(Li et al. 2005)	XVIVO-10	Lonza	
			NEAA	Invitrogen	11140-050
			L-glutamine	Invitrogen	25030-081
			Beta-mercaptoethanol	Invitrogen	21985-023
			hbFGF	RnD Systems	3718-FB
			hFLT3	RnD Systems	308-FKN/CF
2		(Liu et al. 2006)	DMEM/F12	Invitrogen	21041-025
			N2	Invitrogen	17502-048
			B27	Invitrogen	17504-044
			L-glutamine	Invitrogen	25030-081
			Beta-mercaptoethanol	Invitrogen	21985-023
			hbFGF	RnD Systems	3718-FB
3		(Vallier et al. 2005)	IMDM	Invitrogen	21980-32
			F12	Invitrogen	31765-027
			L-glutamine	Invitrogen	25030-081
			Beta-mercaptoethanol	Invitrogen	21985-023
			Insulin	Invitrogen	12585-014
			Transferrin	Invitrogen	11105-012
			Monothioglycerol	Sigma-Aldrich	M6145
			BSA	Europa bioproducts	EQBAC62 - lot BAC62-624
			ActivinA	RnD Systems	338-AC/CF
			hbFGF	RnD Systems	3718-FB
4		(Lu et al. 2006)	DMEM/F12	Invitrogen	21041-025
			L-glutamine	Invitrogen	25030-081
			Beta-mercaptoethanol	Invitrogen	21985-023
			Insulin	Invitrogen	12585-014
			Transferrin	Invitrogen	11105-012
			Cholesterol	Invitrogen	12531-018
			Albumin	Invitrogen	11021-029
			hbFGF	RnD Systems	3718-FB
			WNT3A	RnD Systems	1324-WN/CF
			BAFF	Invitrogen	PHC1674
5		(Yao et al. 2006)	DMEM/F12	Invitrogen	21041-025
			N2	Invitrogen	17502-048
			B27	Invitrogen	17504-044
			NEAA	Invitrogen	11140-050
			L-glutamine	Invitrogen	25030-081
			Beta-mercaptoethanol	Invitrogen	21985-023
			BSA Fraction V	Invitrogen	15260-037
			hbFGF	RnD Systems	3718-FB
6	hESF9	(Furue et al. 2008)	n/a	CSTI	
7	mTeSR1	(Ludwig et al. 2006a)	n/a	Stem Cell Technologies	
8	STEMPRO	(Wang et al. 2007)	n/a	Invitrogen	

Addresses of suppliers:

Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008, USA.

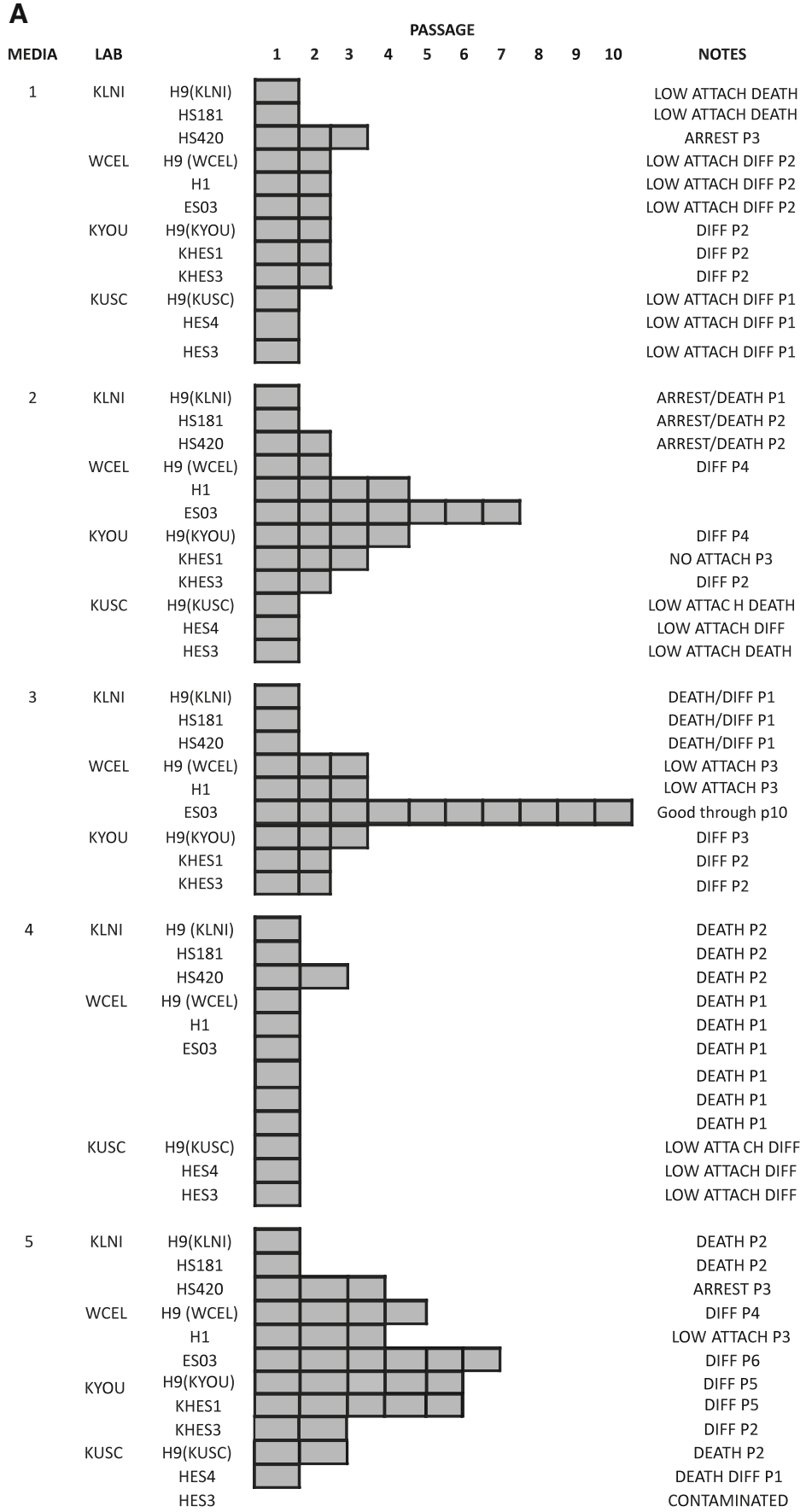
RnD Systems Inc., 614 McKinley Place N.E., Minneapolis, MN 55413, USA.

Stem Cell Technologies 570 West Seventh Avenue, Suite 400, Vancouver, BC, Canada V5Z 1B3

CSTI: Cell Science & Technology Institute, Inc 982-0262 1-chome, Aoba-ku, Sendai, Miyagi, Japan.

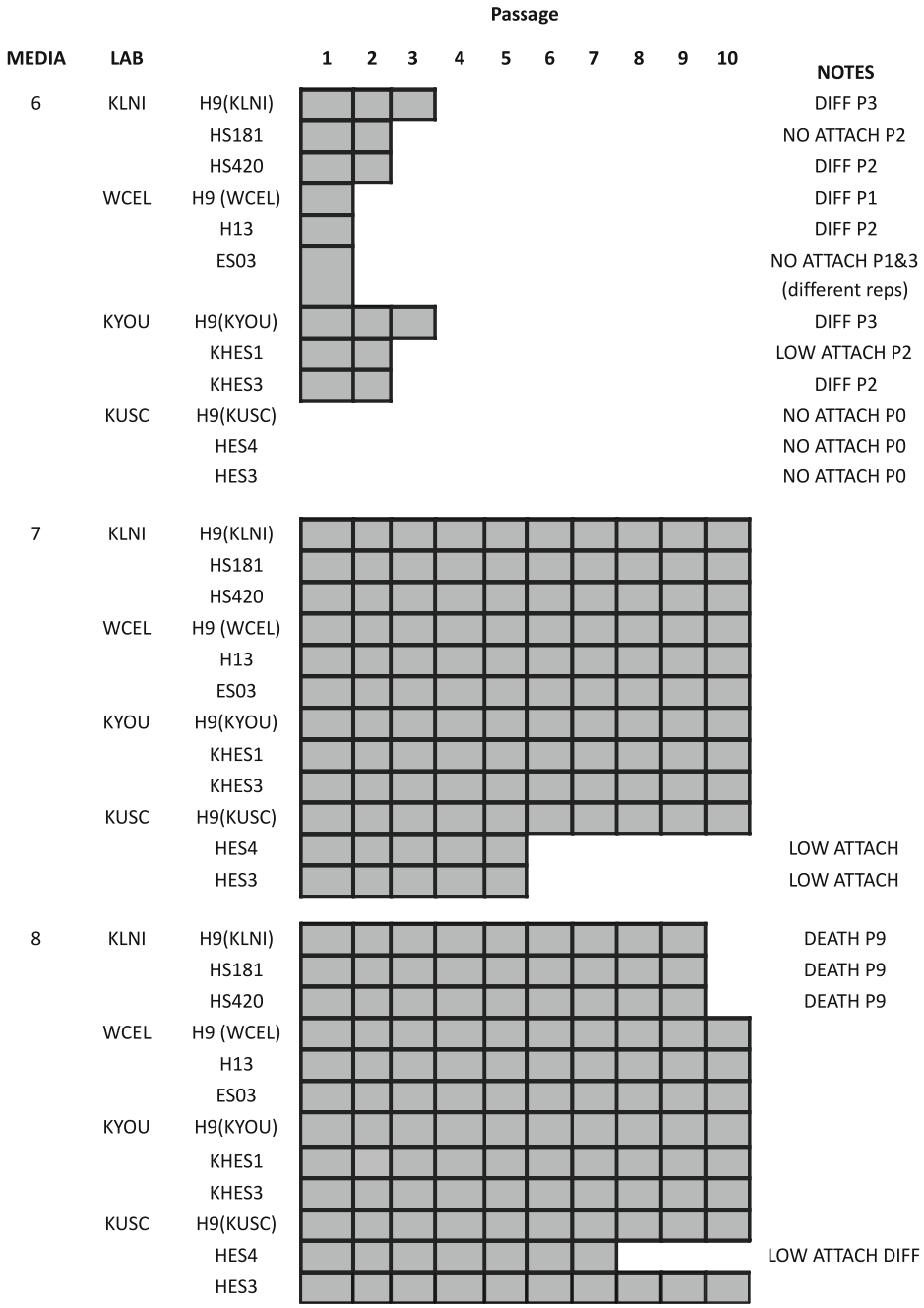
Figure 1. (*A*) Summary test results. (*B*) Results of the retest of the media by UKSCB. For most tests new growth factors were obtained however for testing of medium no. 3 three different batches of Activin A were used. ISCI-GF: Original growth factor batch used in the ISCI study. UKSCB-GF: New Growth factor obtained for the retest. LV-GF: Activin A obtained from the originating laboratory.

A



MEDIA	LAB		NOTES
1	KLNI	H9(KLNI)	LOW ATTACH DEATH
		HS181	LOW ATTACH DEATH
		HS420	ARREST P3
	WCEL	H9 (WCEL)	LOW ATTACH DIFF P2
		H1	LOW ATTACH DIFF P2
		ES03	LOW ATTACH DIFF P2
	KYOU	H9(KYOU)	DIFF P2
		KHES1	DIFF P2
		KHES3	DIFF P2
	KUSC	H9(KUSC)	LOW ATTACH DIFF P1
		HES4	LOW ATTACH DIFF P1
		HES3	LOW ATTACH DIFF P1
2	KLNI	H9(KLNI)	ARREST/DEATH P1
		HS181	ARREST/DEATH P2
		HS420	ARREST/DEATH P2
	WCEL	H9 (WCEL)	DIFF P4
		H1	
		ES03	
	KYOU	H9(KYOU)	DIFF P4
		KHES1	NO ATTACH P3
		KHES3	DIFF P2
	KUSC	H9(KUSC)	LOW ATTAC H DEATH
		HES4	LOW ATTACH DIFF
		HES3	LOW ATTACH DEATH
3	KLNI	H9(KLNI)	DEATH/DIFF P1
		HS181	DEATH/DIFF P1
		HS420	DEATH/DIFF P1
	WCEL	H9 (WCEL)	LOW ATTACH P3
		H1	LOW ATTACH P3
		ES03	Good through p10
	KYOU	H9(KYOU)	DIFF P3
		KHES1	DIFF P2
		KHES3	DIFF P2
4	KLNI	H9 (KLNI)	DEATH P2
		HS181	DEATH P2
		HS420	DEATH P2
	WCEL	H9 (WCEL)	DEATH P1
		H1	DEATH P1
		ES03	DEATH P1
			DEATH P1
			DEATH P1
			DEATH P1
	KUSC	H9(KUSC)	LOW ATTA CH DIFF
		HES4	LOW ATTACH DIFF
		HES3	LOW ATTACH DIFF
5	KLNI	H9(KLNI)	DEATH P2
		HS181	DEATH P2
		HS420	ARREST P3
	WCEL	H9 (WCEL)	DIFF P4
		H1	LOW ATTACH P3
		ES03	DIFF P6
	KYOU	H9(KYOU)	DIFF P5
		KHES1	DIFF P5
		KHES3	DIFF P2
	KUSC	H9(KUSC)	DEATH P2
		HES4	DEATH DIFF P1
		HES3	CONTAMINATED

Figure 1. (continued).

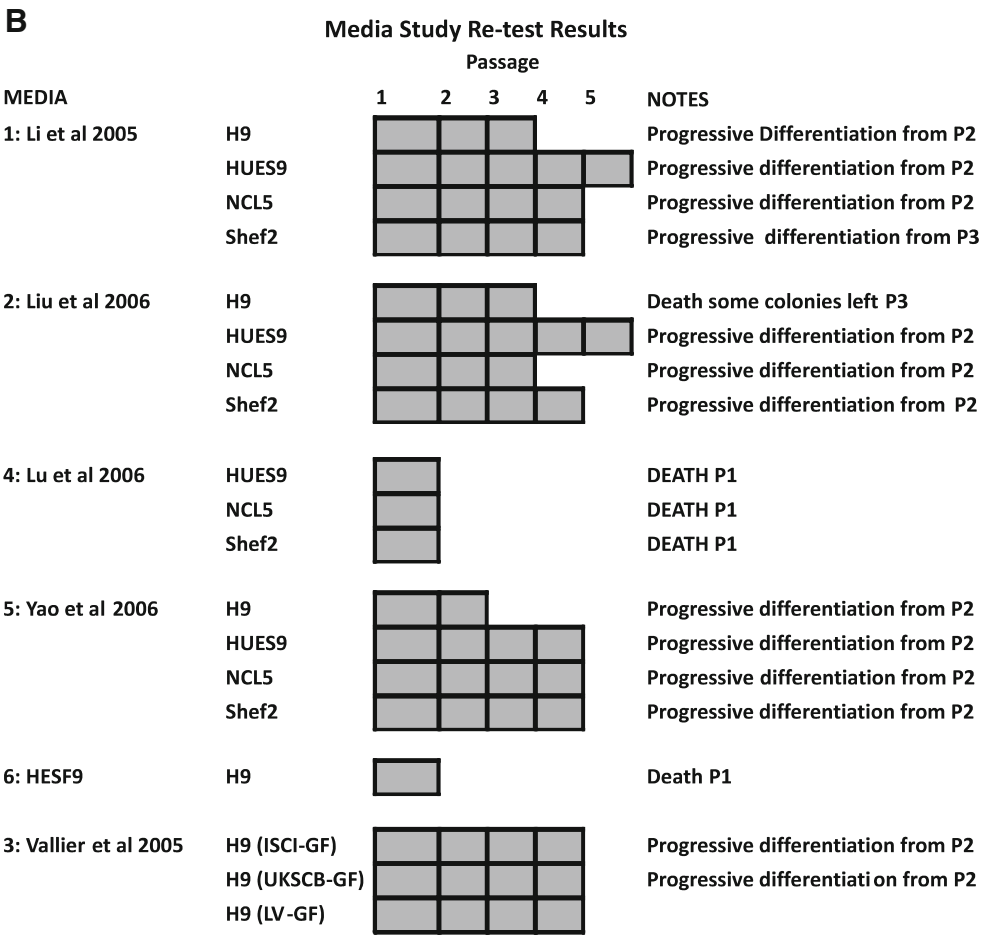


Results

Overall Summary of Attachment, Survival, Growth, and Stem Cell Maintenance. Figure 1*A* displays a chart summarizing the fate of hESC cultures grown in the test media by four laboratories over the course of the study. Results from the four laboratories were generally consistent. Most of the test media failed to support long-term maintenance of stem cell cultures under the conditions of this study. Cultures either failed to initiate or attach in the test media or terminated after passages 2–5 with poor attachment or death or more frequently, morphological appearance of differentiation. By contrast, in all laboratories, mTeSR1 and STEMPRO, and the positive control culture system, all supported stem cell maintenance throughout ten passages. Phase contrast images of hESC colonies that were grown successfully using these media are shown in Fig. 2.

To determine whether or not the failures observed related to a particular batch of test reagents, a fifth laboratory repeated some of the tests on a panel of cell lines using a new set of reagents. The results were similar to those obtained by the four laboratories that originally carried out the study; showing inability of medias 1-6 to support cell line growth beyond a maximum of 5 passages Fig. 1*B*.

Figure 1. (continued).



Interestingly, however, one medium formulation (Vallier et al. 2005), obtained fully supplemented directly from the laboratory of origin with minimal shipment, performed better than either batch formulated by the test laboratory.

Growth Curves. Representative growth curves illustrating results from several different cell lines are shown in Fig. 2. Consistent cell yields were sustained for ten passages only in the control conditions and with the two commercial media, mTeSR1 and STEMPRO. Of the other media, it is notable that no. 2 and no. 5 performed better than some of the others under these conditions.

Flow Cytometry. Quantitative analysis of cell surface antigen expression was carried out at passages 0 and 5 and 10 for those cell lines and culture systems that maintained growth to those time points. Representative data are shown in Fig. 3. The results generally were in line with the overall morphological observations of the cultures and the data on the maintenance of cell numbers. Thus, the positive control and the two commercial defined media supported stem cell maintained expression of stem cell markers. Again the media no. 2 and no. 5 showed maintenance of stem cell markers in some cell lines in some laboratories for up to five passages (data not shown).

Discussion

This study demonstrates that culture of hESC in defined media without feeder cells is not a trivial undertaking even for laboratories with significant experience in the field. Apart from the commercial preparations, most of the formulations did not support maintenance of hESC for even the relatively short period of this study. Retesting of the media that failed to support stem cell maintenance by an independent laboratory, using freshly formulated growth factors, indicated that it was unlikely that the outcomes were due to problems specific to the preparations used in the original studies, but rather to a general difficulty in preparing these media. It is notable however that while the independent laboratory had difficulties with media no. 3, their results were substantially better when they used this medium supplied direct by the laboratory of origin, suggesting a critical need for selecting reagent batches and/or subtleties in media preparation.

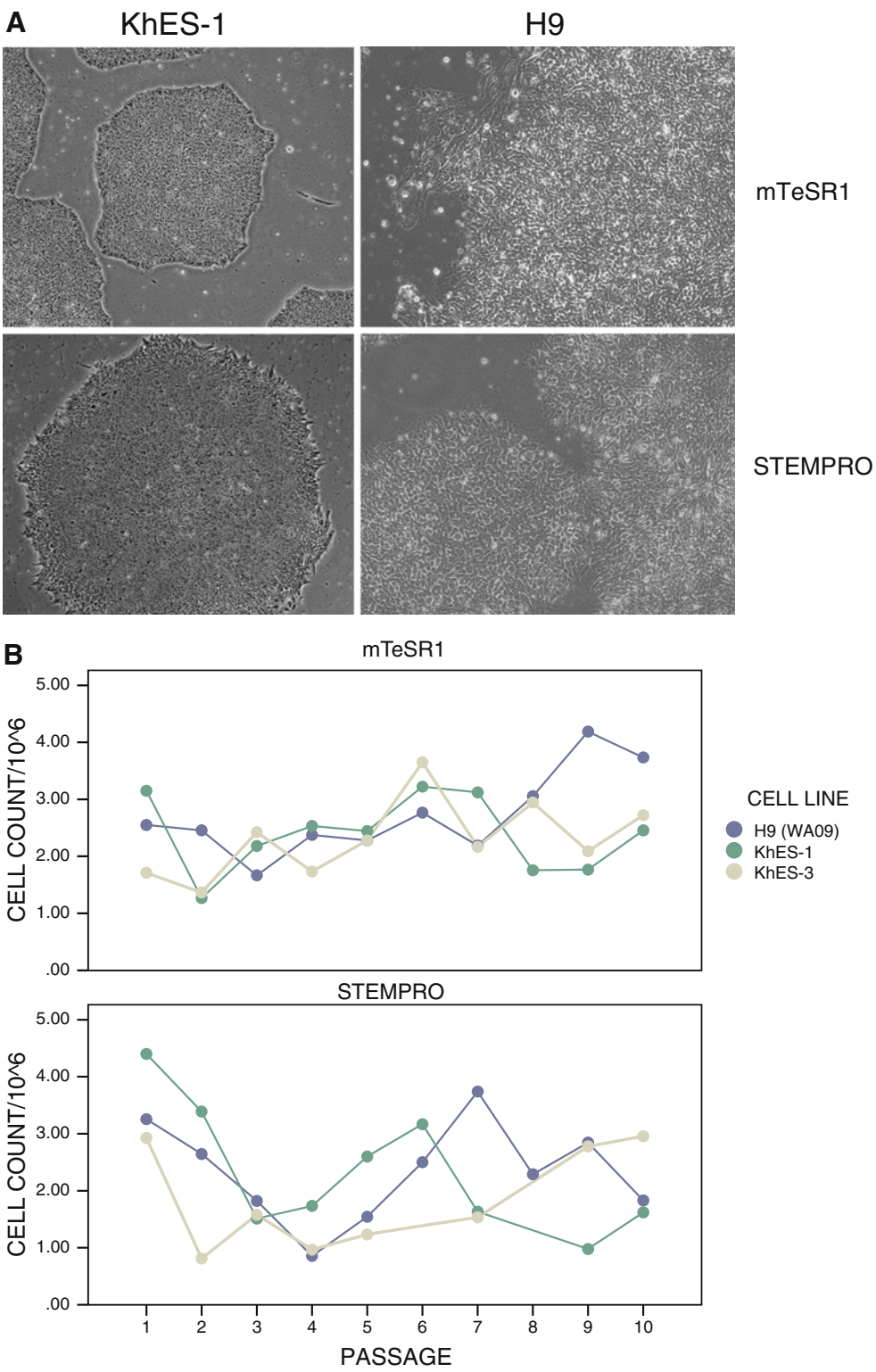


Figure 2. Representative photomicrographs and cell counts. (*A*) Photomicrographs of KhES-1 and H9 (WA09) respectively grow to ten passages in mTeSR1 and STEMPRO, respectively. (*B*) Representative cell counts from each passage for the cell lines WA09 (H9), KhES-1, and KhES-3.

It is now well established that both FGF and Activin/nodal/TGF-beta signaling are critical for hESC maintenance (Beattie et al. 2005; James et al. 2005; Vallier et al. 2005, 2009; Xiao et al. 2006; Greber et al. 2007; Xu et al. 2008). Of the formulations tested, only TeSR, Stem Pro, and no. 3 contain agonists for both pathways: of these, the two commercial media, mTeSR1 and STEM PRO, were the most successful in supporting stem cell growth. These commercial media also contained agonists of other signaling systems, such as GABA receptors and ErbB2, which

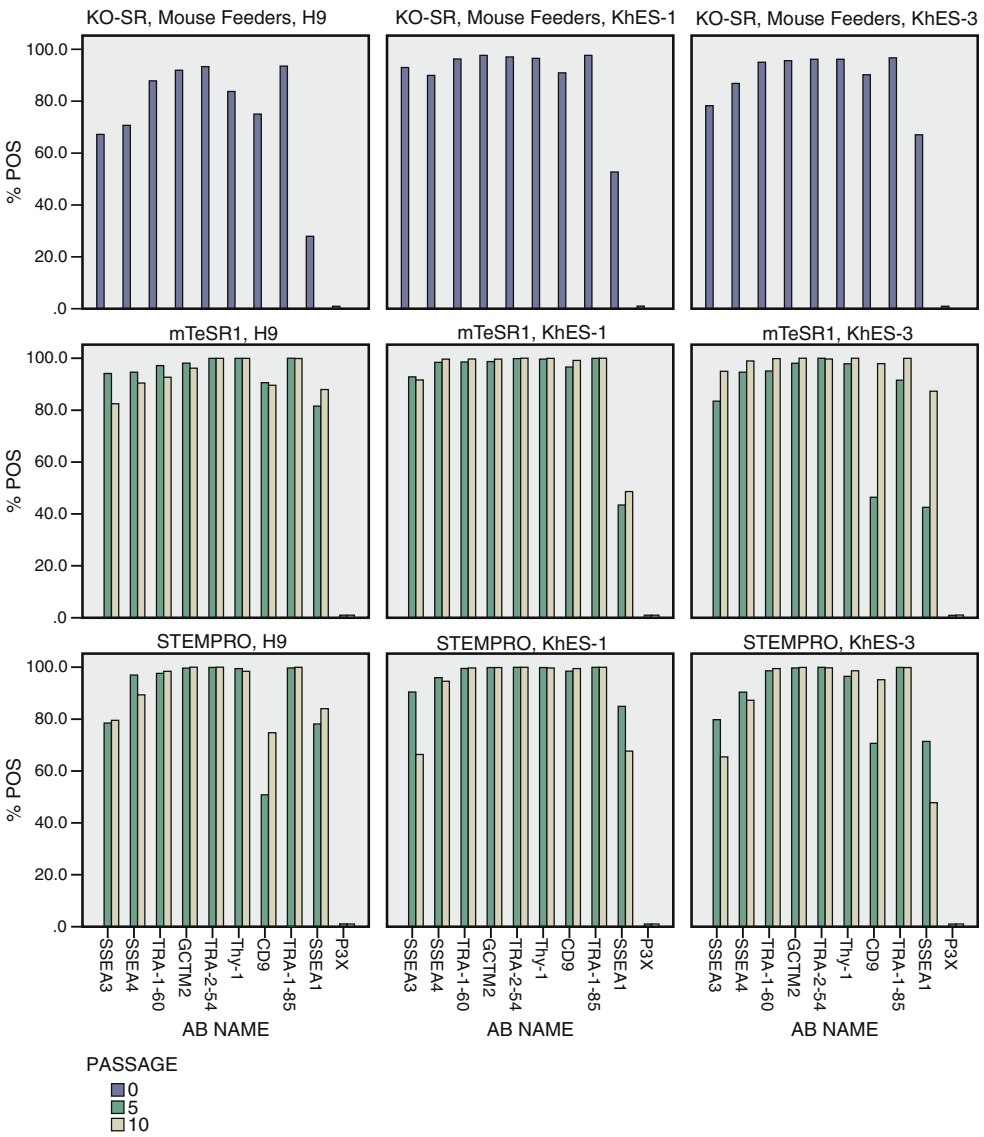


Figure 3. Representative flow cytometry data. Representative flow cytometry data expressed as percentage of cells called positive for three cell lines H9, KhES-1, and KhES-3 at passage 0 in Knockout Serum Replacer, FGF-2, and mouse embryonic fibroblast feeder cells and at passage 5 and 10 for cells grown in mTeSR1 and STEMPRO.

while less well characterized, are thought to play a role in hESC maintenance.

Considerable effort in development, manufacture, and quality control goes into the formulation and production of commercial media, and it may be that it is difficult to implement equivalent reproducibility in the context of an academic laboratory, particularly when transferring a medium preparation process to many laboratories. This factor might account for the relative success of the commercial preparations observed in our study. On the other hand, there are ample reports of successful long-term propagation of hESC in media supplemented with FGF-2, or other reagents, from a number of different academic laboratories, and the only preparation required of the test laboratories in this study was addition of factors and a few other components to manufactured basal media.

Another explanation for the variability of outcomes observed here compared to published results might lie in the hESC themselves. hESC produce a number of polypeptides that can influence their growth and differentiation. Both FGF and nodal are expressed in hESC cultures (Sperger et al. 2003; Sato et al. 2003; Ginis et al. 2004), as are antagonists of nodal/activin signaling (Brandenberger et al. 2004), as well as BMPs, which activate SMAD1/5/8 signaling to drive hESC differentiation (Sato et al. 2003). Differences in production of such factors might reflect subtle differences in methods of passaging (e.g., sizes of cell clumps that are passaged), which are difficult to standardize between laboratories or glean from published details. It is also possible that epigenetic adaptation in vitro leads to modulation of the activity of these pathways in hESC cultures, and that this process varies between different cell lines, or even between the same cell line maintained in different laboratories. In particular genotypic and epigenetic differences between cell lines may give rise to differences in the expression levels of different receptor subtypes such as

FGF and TGF-beta receptors and cell attachment modulators such as the Integrin family of receptors. Changes in cell surface receptor expression and cell adhesion modulators between cell lines or indeed at different phases of the hESC culture regrowth may necessitate that different hESC isolates have quite distinct exogenous factor requirements in a given growth media to achieve proper cell adhesion and maintenance of the undifferentiated state. It would be possible to compare the endogenous activity of some of these critical signaling pathways between ES cell lines and sublines and then relate this to dependence upon exogenous factors.

Whatever the role of endogenous autocrine or paracrine-signaling in hESC maintenance, the activation of multiple pathways driving stem cell maintenance and the inhibition of pathways that drive differentiation by combinations of agonists/antagonists with distinct mechanisms of action represent a robust strategy for development of defined culture systems.

Conclusions

Eight different defined culture systems were assessed in a multicenter study for their ability to support hESC maintenance for ten passages. Two commercial media, mTeSR1 and STEMPRO, consistently functioned well in assays of growth and stem cell maintenance throughout the duration of the study.

Acknowledgments This study was supported by funding from the International Stem Cell Forum (www.stemcellforum.org). In addition, further support for the Test Laboratory at the Karolinska Institute (Hovatta and Holm) by ESTOOLS, a Six Framework Integrated Project funded by the European Union. In addition, at the Test Laboratory at WiCell (Ludwig and Brehm) this project was funded in whole or in part with Federal funds from the National Center for Research Resources, National Institutes of Health, Department of Health and Human Services, under Contract no. HHSN309200582085C. The content of this publication does not necessarily reflect the views or policies of the U.S. Department of Health and Human Services nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. We thank all member laboratories of the International Stem Cell Initiative for their input into the design of the study. The media mTeSR1 and hESF9 were supplied free of charge by StemCell Technologies Inc., Canada, and Cell Science & Technology Institute, Inc., Japan (CSTI), respectively. WiCell, USA provided cultures of WA09 hESC. The study design was initiated and discussed at international meetings of the ISCI held at The Jackson Laboratory, Bar Harbor, ME, USA.

References

Adewumi O.; Aflatoonian B.; Ahrlund-Richter L.; Amit M.; Andrews P. W.; Beighton G.; Bello P. A.; Benvenisty N.; Berry L. S.; Bevan S. et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat. Biotechnol.* 25: 803–816; 2007.

Amit M.; Itskovitz-Eldor J. Maintenance of human embryonic stem cells in animal serum- and feeder layer-free culture conditions. *Methods Mol. Biol.* 331: 105–113; 2006.

Andrews P. W.; Benvenisty N.; McKay R.; Pera M. F.; Rossant J.; Semb H.; Stacey G. N. The International Stem Cell Initiative: toward benchmarks for human embryonic stem cell research. *Nat. Biotechnol.* 23: 795–797; 2005.

Andrews P. W.; Goodfellow P. N.; Shevinsky L. H.; Bronson D. L.; Knowles B. B. Cell-surface antigens of a clonal human embryonal carcinoma cell line: morphological and antigenic differentiation in culture. *Int. J. Cancer* 29: 523–531; 1982.

Beattie G. M.; Lopez A. D.; Bucay N.; Hinton A.; Firpo M. T.; King C. C.; Hayek A. Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells* 23: 489–495; 2005.

Brandenberger R.; Wei H.; Zhang S.; Lei S.; Murage J.; Fisk G. J.; Li Y.; Xu C.; Fang R.; Guegler K. et al. Transcriptome characterization elucidates signaling networks that control human ES cell growth and differentiation. *Nat. Biotechnol.* 22: 707–716; 2004.

Chase L. G.; Firpo M. T. Development of serum-free culture systems for human embryonic stem cells. *Curr Opin Chem Biol* 11: 367–372; 2007.

Furue M. K.; Na J.; Jackson J. P.; Okamoto T.; Jones M.; Baker D.; Hata R.; Moore H. D.; Sato J. D.; Andrews P. W. Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc. Natl. Acad. Sci. U. S. A.* 105: 13409–13414; 2008.

Ginis I.; Luo Y.; Miura T.; Thies S.; Brandenberger R.; Gerecht-Nir S.; Amit M.; Hoke A.; Carpenter M. K.; Itskovitz-Eldor J.; Rao M. S. Differences between human and mouse embryonic stem cells. *Dev. Biol.* 269: 360–380; 2004.

Greber B.; Lehrach H.; Adjaye J. Fibroblast growth factor 2 modulates transforming growth factor beta signaling in mouse embryonic fibroblasts and human ESCs (hESCs) to support hESC self-renewal. *Stem Cells* 25: 455–464; 2007.

James D.; Levine A. J.; Besser D.; Hemmati-Brivanlou A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* 132: 1273–1282; 2005.

Josephson R.; Ording C. J.; Liu Y.; Shin S.; Lakshmipathy U.; Toumadje A.; Love B.; Chesnut J. D.; Andrews P. W.; Rao M. S.; Auerbach J. M. Qualification of embryonal carcinoma 2102Ep as a reference for human embryonic stem cell research. *Stem Cells* 25: 437–446; 2007.

Klein P. S.; Melton D. A. A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. U. S. A.* 93: 8455–8459; 1996.

Li Y.; Powell S.; Brunette E.; Lebkowski J.; Mandalam R. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. *Biotechnol. Bioeng.* 91: 688–698; 2005.

Liu Y.; Song Z.; Zhao Y.; Qin H.; Cai J.; Zhang H.; Yu T.; Jiang S.; Wang G.; Ding M.; Deng H. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. *Biochem. Biophys. Res. Commun.* 346: 131–139; 2006.

Lu J.; Hou R.; Booth C. J.; Yang S. H.; Snyder M. Defined culture conditions of human embryonic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 103: 5688–5693; 2006.

Ludwig T. E.; Bergendahl V.; Levenstein M. E.; Yu J.; Probasco M. D.; Thomson J. A. Feeder-independent culture of human embryonic stem cells. *Nat Methods* 3: 637–646; 2006a.

Ludwig T. E.; Levenstein M. E.; Jones J. M.; Berggren W. T.; Mitchen E. R.; Frane J. L.; Crandall L. J.; Daigh C. A.; Conard K. R.; Piekarczyk M. S. et al. Derivation of human embryonic stem cells in defined conditions. *Nat. Biotechnol.* 24: 185–187; 2006b.

Reubinoff B. E.; Pera M. F.; Fong C. Y.; Trounson A.; Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.* 18: 399–404; 2000.

Sato N.; Sanjuan I. M.; Heke M.; Uchida M.; Naef F.; Brivanlou A. H. Molecular signature of human embryonic stem cells and its comparison with the mouse. *Dev. Biol.* 260: 404–413; 2003.

Sperger J. M.; Chen X.; Draper J. S.; Antosiewicz J. E.; Chon C. H.; Jones S. B.; Brooks J. D.; Andrews P. W.; Brown P. O.; Thomson J. A. Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proc. Natl. Acad. Sci. U. S. A.* 100: 13350–13355; 2003.

Thomson J. A.; Itskovitz-Eldor J.; Shapiro S. S.; Waknitz M. A.; Swiergiel J. J.; Marshall V. S.; Jones J. M. Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147; 1998.

Unger C.; Skottman H.; Blomberg P.; Dilber M. S.; Hovatta O. Good manufacturing practice and clinical-grade human embryonic stem cell lines. *Hum. Mol. Genet.* 17: R48–R53; 2008.

Vallier L.; Alexander M.; Pedersen R. A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J. Cell Sci.* 118: 4495–4509; 2005.

Vallier L.; Mendjan S.; Brown S.; Chng Z.; Teo A.; Smithers L. E.; Trotter M. W.; Cho C. H.; Martinez A.; Rugg-Gunn P. et al. Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. *Development* 136: 1339–1349; 2009.

Wang L.; Schulz T. C.; Sherrer E. S.; Dauphin D. S.; Shin S.; Nelson A. M.; Ware C. B.; Zhan M.; Song C. Z.; Chen X. et al. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. *Blood* 110: 4111–4119; 2007.

Xiao L.; Yuan X.; Sharkis S. J. Activin A maintains self-renewal and regulates fibroblast growth factor, Wnt, and bone morphogenic protein pathways in human embryonic stem cells. *Stem Cells* 24: 1476–1486; 2006.

Xu R. H.; Sampsell-Barron T. L.; Gu F.; Root S.; Peck R. M.; Pan G.; Yu J.; Antosiewicz-Bourget J.; Tian S.; Stewart R.; Thomson J. A. NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. *Cell Stem Cell* 3: 196–206; 2008.

Yao S.; Chen S.; Clark J.; Hao E.; Beattie G. M.; Hayek A.; Ding S. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc. Natl. Acad. Sci. U. S. A.* 103: 6907–6912; 2006.