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.

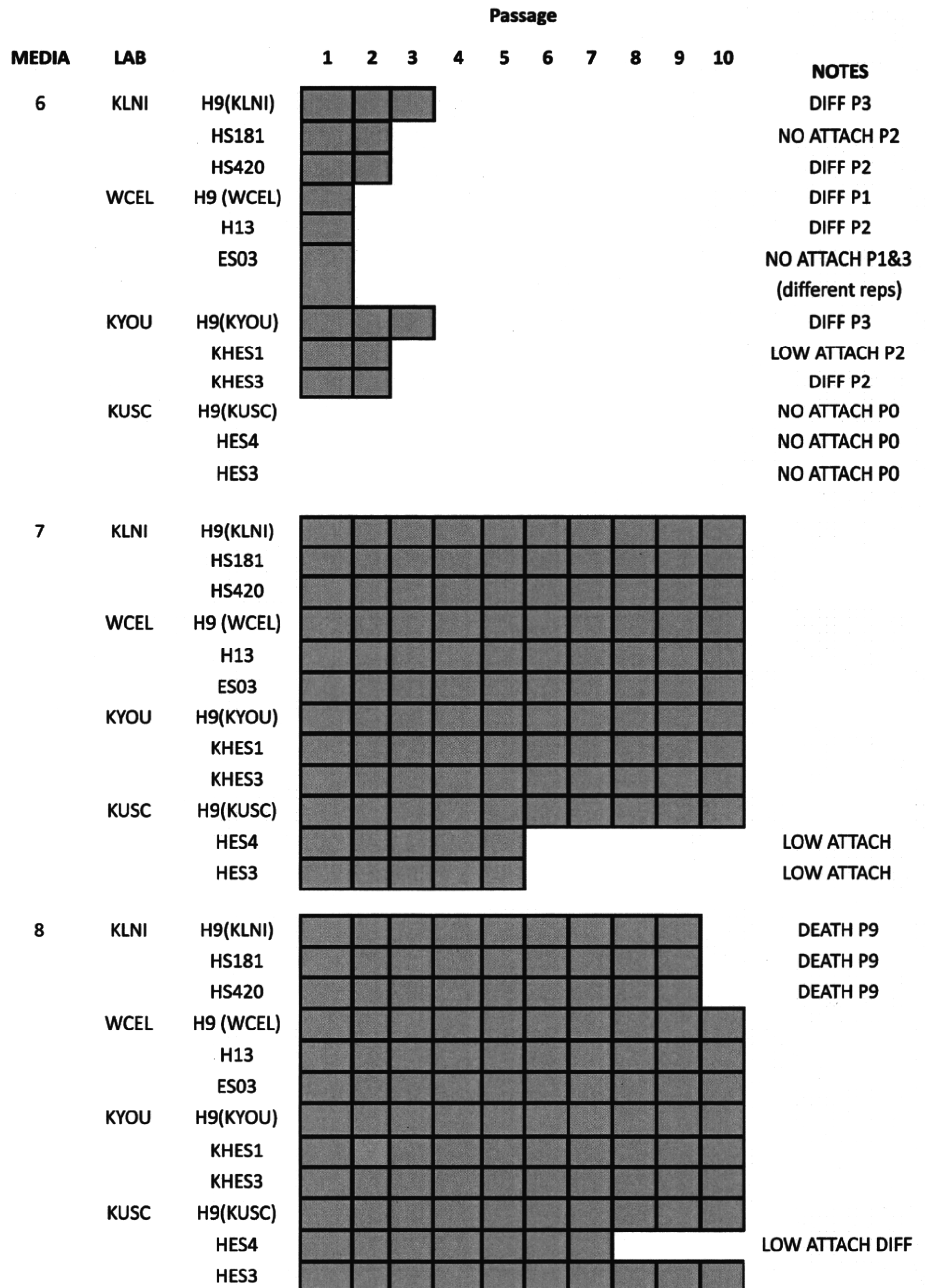
Figure 1. (*A*) Summary test results. (*B*) Results of the retest of the media by UKSCB. For most tests new growth factors were obtained however for testing of medium no. 3 three different batches of Activin A were used. ISCI-GF: Original growth factor batch used in the ISCI study. UKSCB-GF: New Growth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

A

PASSAGE 1 2 3 4 5 6 7 8 9 10

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).

B

Media Study Re-test Results

MEDIA		Passage 1	2	3	4	5	NOTES
1: Li et al 2005	H9	■	■	■			Progressive Differentiation from P2
	HUES9	■	■	■	■	■	Progressive differentiation from P2
	NCL5	■	■	■	■		Progressive differentiation from P2
	Shef2	■	■	■	■		Progressive differentiation from P3
2: Liu et al 2006	H9	■	■	■			Death some colonies left P3
	HUES9	■	■	■	■	■	Progressive differentiation from P2
	NCL5	■	■	■			Progressive differentiation from P2
	Shef2	■	■	■	■		Progressive differentiation from P2
4: Lu et al 2006	HUES9	■					DEATH P1
	NCL5	■					DEATH P1
	Shef2	■					DEATH P1
5: Yao et al 2006	H9	■	■				Progressive differentiation from P2
	HUES9	■	■	■	■		Progressive differentiation from P2
	NCL5	■	■	■	■		Progressive differentiation from P2
	Shef2	■	■	■	■		Progressive differentiation from P2
6: HESF9	H9	■					Death P1
3: Vallier et al 2005	H9 (ISCI-GF)	■	■	■	■		Progressive differentiation from P2
	H9 (UKSCB-GF)	■	■	■	■		Progressive differentiati on from P2
	H9 (LV-GF)	■	■	■	■		

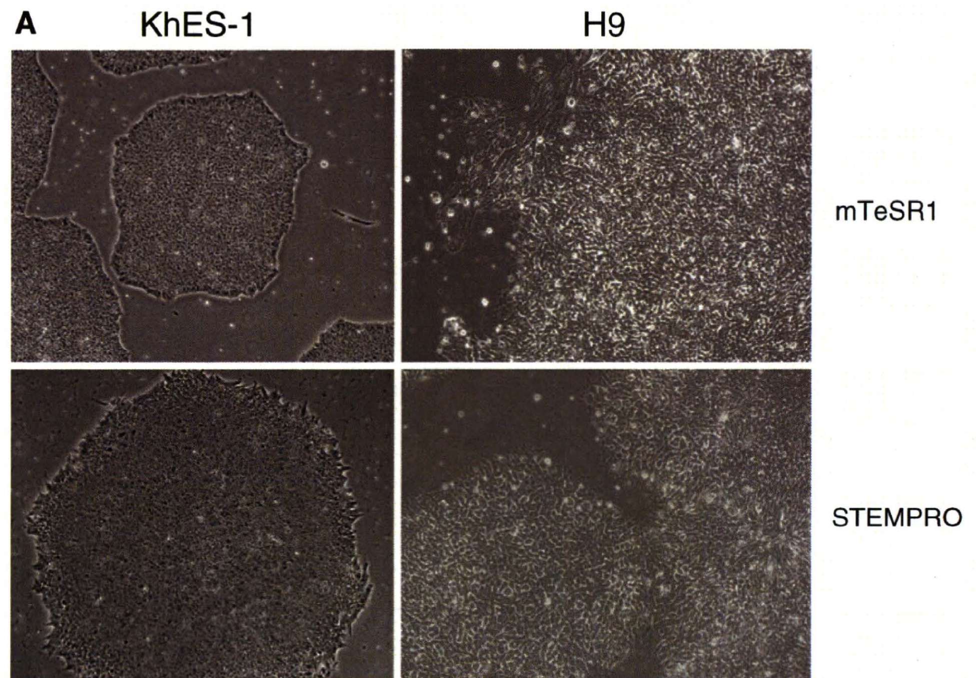
Interestingly, however, one medium formulation (Vallier et al. 2005), obtained fully supplemented directly from the laboratory of origin with minimal shipment, performed better than either batch formulated by the test laboratory.

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.



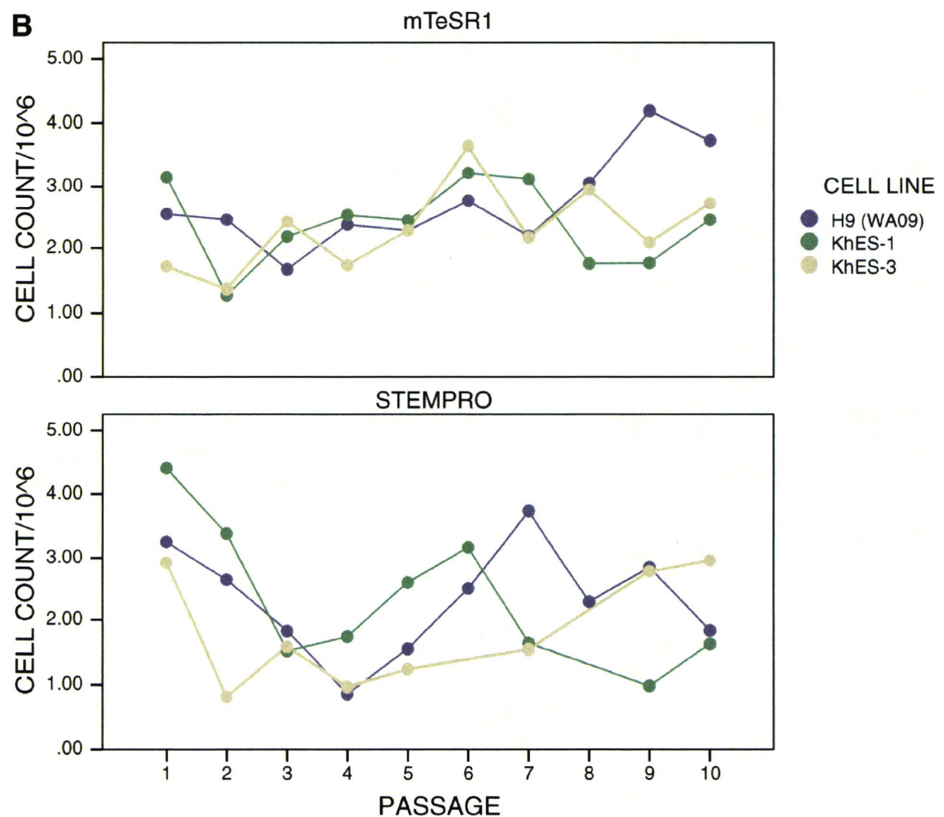


Figure 2. Representative photomicrographs and cell counts. (*A*) Photomicrographs of KhES-1 and H9 (WA09) respectively grow to ten passages in mTeSR1 and STEMPRO, respectively. (*B*) Representative cell counts from each passage for the cell lines WA09 (H9), KhES-1, and KhES-3.

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

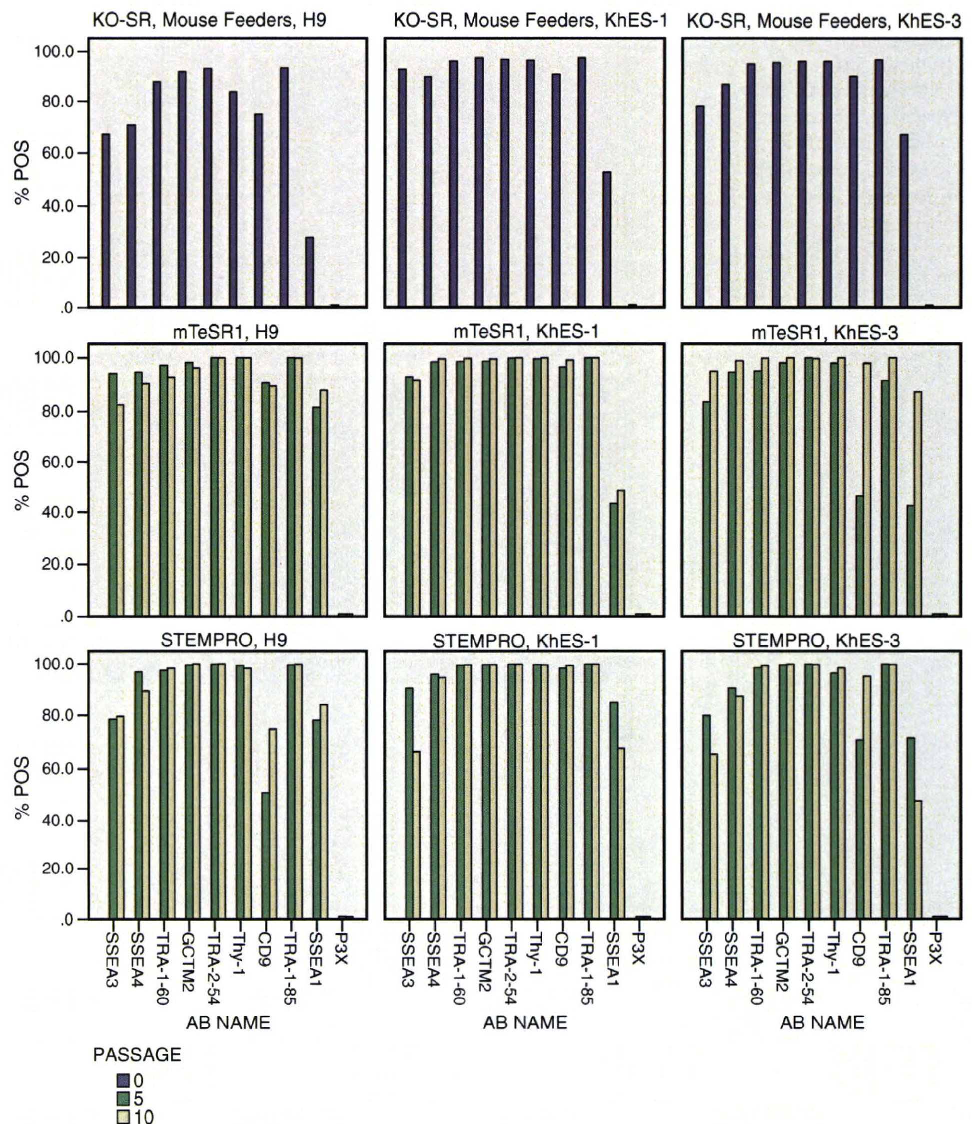


Figure 3. Representative flow cytometry data. Representative flow cytometry data expressed as percentage of cells called positive for three cell lines H9, KhES-1, and KhES-3 at passage 0 in Knockout Serum Replacer, FGF-2, and mouse embryonic fibroblast feeder cells and at passage 5 and 10 for cells grown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

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Biochemical and Biophysical Research Communications 401 (2010) 480–486

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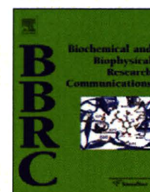

Chemical inhibition of sulfation accelerates neural differentiation of mouse embryonic stem cells and human induced pluripotent stem cells

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ARTICLE INFO

Article history:
Received 12 September 2010
Available online 25 September 2010

Keywords:
Chlorate
Sulfation
Neural differentiation
Mouse embryonic stem cell
Human induced pluripotent stem cell

ABSTRACT

Pluripotency of embryonic stem cells (ESCs) is maintained by the balancing of several signaling pathways, such as Wnt, BMP, and FGF, and differentiation of ESCs into a specific lineage is induced by the disruption of this balance. Sulfated glycans are considered to play important roles in lineage choice of ESC differentiation by regulating several signalings. We examined whether reduction of sulfation by treatment with the chemical inhibitor chlorate can affect differentiation of ESCs. Chlorate treatment inhibited mesodermal differentiation of mouse ESCs, and then induced ectodermal differentiation and accelerated further neural differentiation. This could be explained by the finding that several signaling pathways involved in the induction of mesodermal differentiation (Wnt, BMP, and FGF) or inhibition of neural differentiation (Wnt and BMP) were inhibited in chlorate-treated embryoid bodies, presumably due to reduced sulfation on heparan sulfate and chondroitin sulfate. Furthermore, neural differentiation of human induced pluripotent stem cells (hiPSCs) was also accelerated by chlorate treatment. We propose that chlorate could be used to induce efficient neural differentiation of hiPSCs instead of specific signaling inhibitors, such as Noggin.

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1. Introduction

Embryonic stem cells (ESCs) [1–3] are promising tools for biotechnology and possess key features that should allow their exploitation in the development of cell replacement therapies [4]. Extrinsic signaling pathways are key mechanisms for determining ESC cell fate, and sulfated glycans, such as heparan sulfate (HS), are well known regulators of signal transduction [5]. HS chains are present abundantly on the cell surface of undifferentiated mouse ESCs (mESCs) and functional roles of HS chains have been demonstrated [6,7]. Thus, the modification of sulfated glycans is an attractive approach for developing methods to regulate ESC differentiation.

Sulfated glycans are synthesized in the Golgi as follows. The high energy sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS), which is required as a substrate for sulfation, is synthesized in the cytosol and nucleus by PAPS synthetase [8], and subsequently is translocated via the PAPS transporter (PAPST) into the Golgi [9–13], where it is used by sulfotransferases to sulfate glycans. Recently, we have demonstrated that the reduction of sulfation by knockdown of *PAPST* using vector-based RNA interference (RNAi) promotes neurogenesis of mESCs [13]. However, the rapid, simple, and safety method for modification of sulfated glycans instead of gene transfer is desired particularly for application of human ESCs (hESCs) to regenerative medicine.

Chlorate inhibits ATP sulfurylase activity of PAPS synthetases by competing with sulfate ions in binding to ATP-sulfurylase resulting in reduction of PAPS [14]. Thus, inhibition of PAPS synthesis by chlorate leads to reduction of sulfation on several sulfated proteins, glycoproteins, glycolipids, and proteoglycans [15,16].

Since induced pluripotent stem cells (iPSCs) were reported [17], the application of human iPSCs (hiPSCs) to regenerative medicine has been expected. In particular, neural induction of hiPSCs is an important research tool for several neural diseases and has been applied to cell replacement therapies. Therefore, methods of efficient and rapid neural induction are required [18]. In this study, we examined whether neural differentiation of hiPSCs in addition to mESCs was enhanced by the chemical down-regulation of sulfation with chlorate.

2. Materials and methods

2.1. Cell culture

R1 [19] and E14TG2a [20] mESC lines were maintained on mouse embryonic fibroblasts (MEFs) inactivated with 10 μg/ml

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doi:10.1016/j.bbrc.2010.09.085

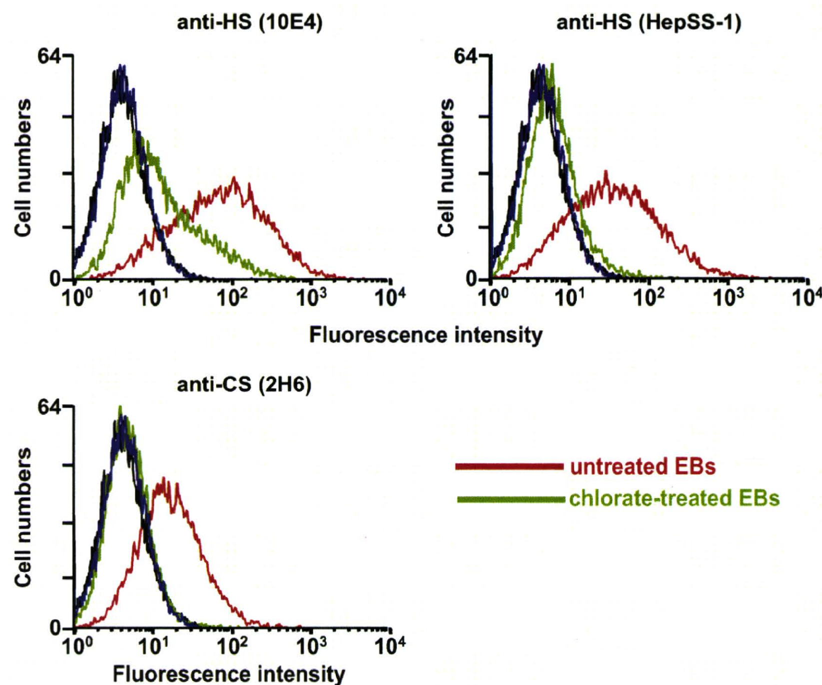

Fig. 1. Chlorate treatment reduces sulfation of HS and CS in EBs. FACS analysis of cells 3 days after EB formation using an anti-HS antibody (10E4 or HepSS-1) or an anti-CS antibody (2H6) (black and blue lines represent the IgM isotype control for untreated and chlorate-treated EBs, respectively). Chlorate treatment was performed for 24 h from 2 days after EB formation. Three independent experiments were performed and representative results are shown.

mitomycin C (Sigma) in ESC medium (DMEM supplemented with 15% FBS {Hyclone}, 1% penicillin/streptomycin {Gibco}, 0.1 mM 2-mercaptoethanol {Gibco}, and 0.1 mM non-essential amino acids {Gibco}) with 1000 U/ml LIF (Chemicon). hiPSC clones (MRC-hiPS_Fetch {NIHS0604} and MRC-hiPS_Tic {JCRB1331}) [21] were maintained on inactivated MEFs in iPSellon (Cell-Sight) with 10 ng/ml bFGF (Wako). All mESC experiments were performed using the R1 line and most results were confirmed using the E14TG2a line.

For embryoid body (EB) formation, mESCs or hiPSCs were transferred to low cell binding 60 mm dishes (Nunc) and cultured in ESC medium without LIF or iPSellon without bFGF, respectively. Before EB formation, hiPSCs were preplated on gelatin-coated dishes to remove feeder cells. For neuronal differentiation, 1 μM all-trans retinoic acid (RA) (Sigma) was added 4 days after EB formation. Then, 5 days after EB formation, EBs were plated onto PDL/laminin-coated 60 mm dishes (Becton Dickinson) in DMEM-F12 containing N2 supplement (Gibco).

To down-regulate sulfation, 50 mM chlorate (Sigma) was added from 2 days after EB formation throughout EB culture.

2.2. FACS analysis

FACS analysis was performed 3 days after EB formation. After EDTA treatment, the cell suspension was incubated with primary antibodies diluted in FACS buffer (0.5% bovine serum albumin and 0.1% sodium azide in PBS). After washing, the cell suspension was incubated with FITC-conjugated secondary antibody (Sigma) diluted in FACS buffer. Cell sorting and analysis were performed using a FACSAria Cell Sorter (Becton Dickinson). Primary antibodies were as follows: mouse IgM isotype control (Chemicon), the anti-HS antibody 10E4 (Seikagaku Corp.), the anti-HS antibody HepSS-1 (Seikagaku Corp.), the anti-chondroitin sulfate (CS) antibody 2H6 (Seikagaku Corp.).

2.3. Immunoblotting

Cells were lysed with lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, protease inhibitors). Isolation of nuclear extracts was performed as described previously [6]. Samples containing 5 μg of cell lysate or nuclear extract were separated by 10% SDS–PAGE and transferred onto PVDF membranes (Millipore). After blocking, the membranes were incubated with primary antibodies as follows: anti-ERK1/2 (Cell Signaling Technology), anti-phosphorylated ERK1/2 (Thr183/185; Cell Signaling Technology), anti-phosphorylated Smad1 (Ser463/465; Cell Signaling Technology), anti-β-actin (Sigma), anti-β-catenin (Cell Signaling Technology), anti-lamin B_1 (Zymed), and anti-βIII-tubulin (Chemicon). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology), washed and developed with ECL Plus reagents (GE Healthcare).

2.4. Immunostaining

After neural differentiation on PLL/laminin-coated glass chamber slides (Iwaki), cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. After washing and subsequent blocking, cells were stained with an anti-βIII-Tubulin antibody. After washing, cells were stained with an FITC-conjugated secondary antibody and counterstained with propidium iodide (PI). Immunofluorescence images were obtained using an LSM5Pascal confocal laser scanning microscope (Carl Zeiss).

2.5. Real-time PCR

Real-time PCR was performed as described previously [6]. For hiPSCs, FastStart Universal SYBR Green Master (Roche) was used. Primer sets and probes are listed in Supplementary Table 1.

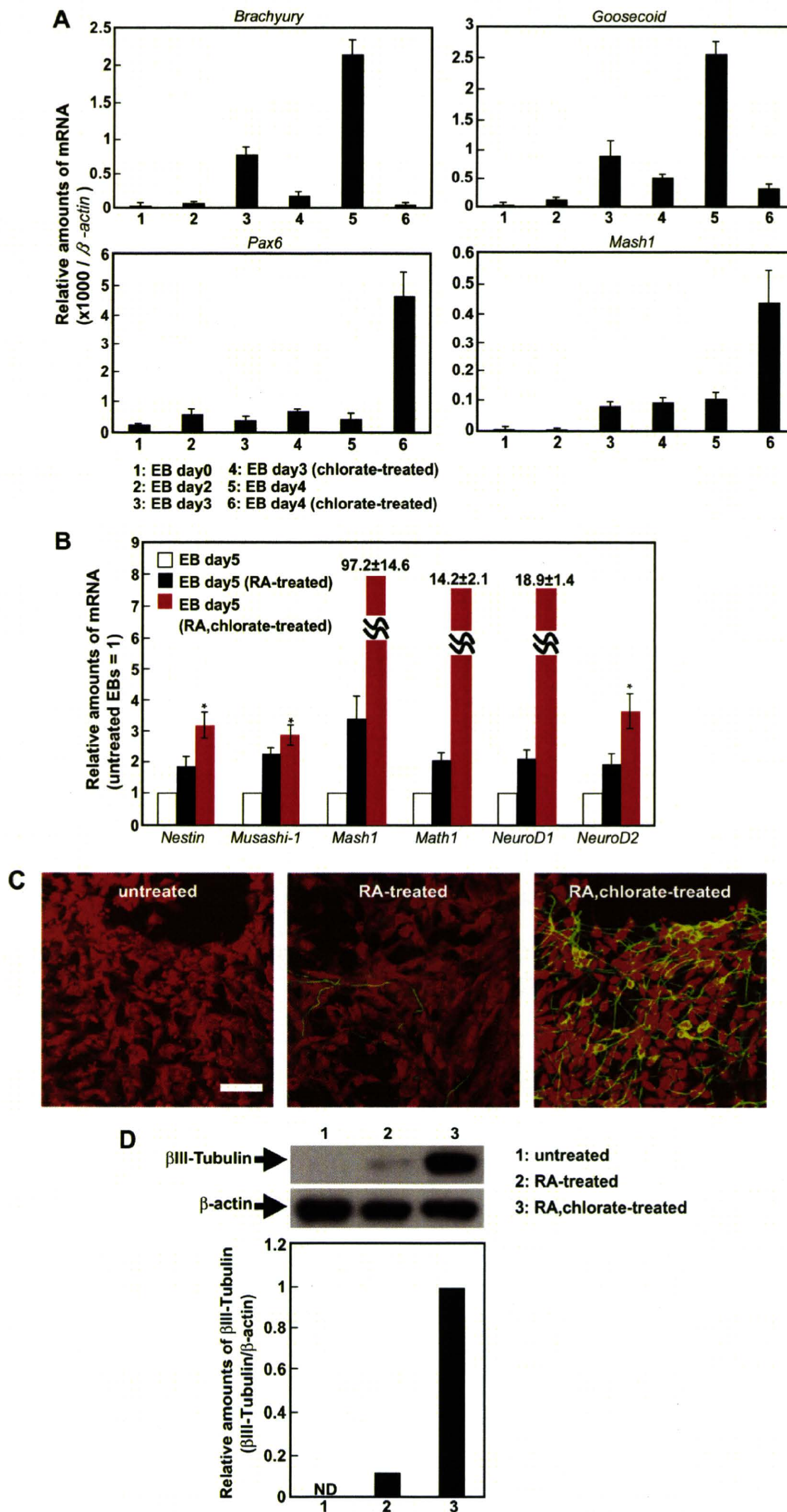

Fig. 2. Reduction of sulfation accelerates neural differentiation of mESCs. (A) Real-time PCR analysis of markers of mesoderm (*Brachyury*, *Goosecoid*) and ectoderm (*Mash1*, *Pax6*) in EBs harvested on each day shown. Relative amounts of each mRNA were calculated after normalization to *β-actin* mRNA. The values shown are the means ± SD of three independent experiments. (B) Real-time PCR analysis of neural markers, such as neural stem/progenitor cell markers (*Nestin*, *Musashi-1*) and proneural markers (*Mash1*, *Math1*, *NeuroD1* and *NeuroD2*), 5 days after EB formation. The results are shown after normalization against the values obtained with untreated EBs (value = 1). The values shown are the means ± SD of duplicate measurements from two independent experiments and significant values are indicated; $^{*}P < 0.05$, compared with the RA-treated EBs. (C) Immunocytochemical staining 2 days after replating of EBs. Representative confocal images from two independent experiments are shown (βIII-tubulin, green; PI, red). Scale bar, 20 μm. (D) Western blotting 2 days after replating of EBs. Representative immunoblots from two independent experiments are shown. The histogram shows mean densitometric readings of βIII-tubulin/loading controls.

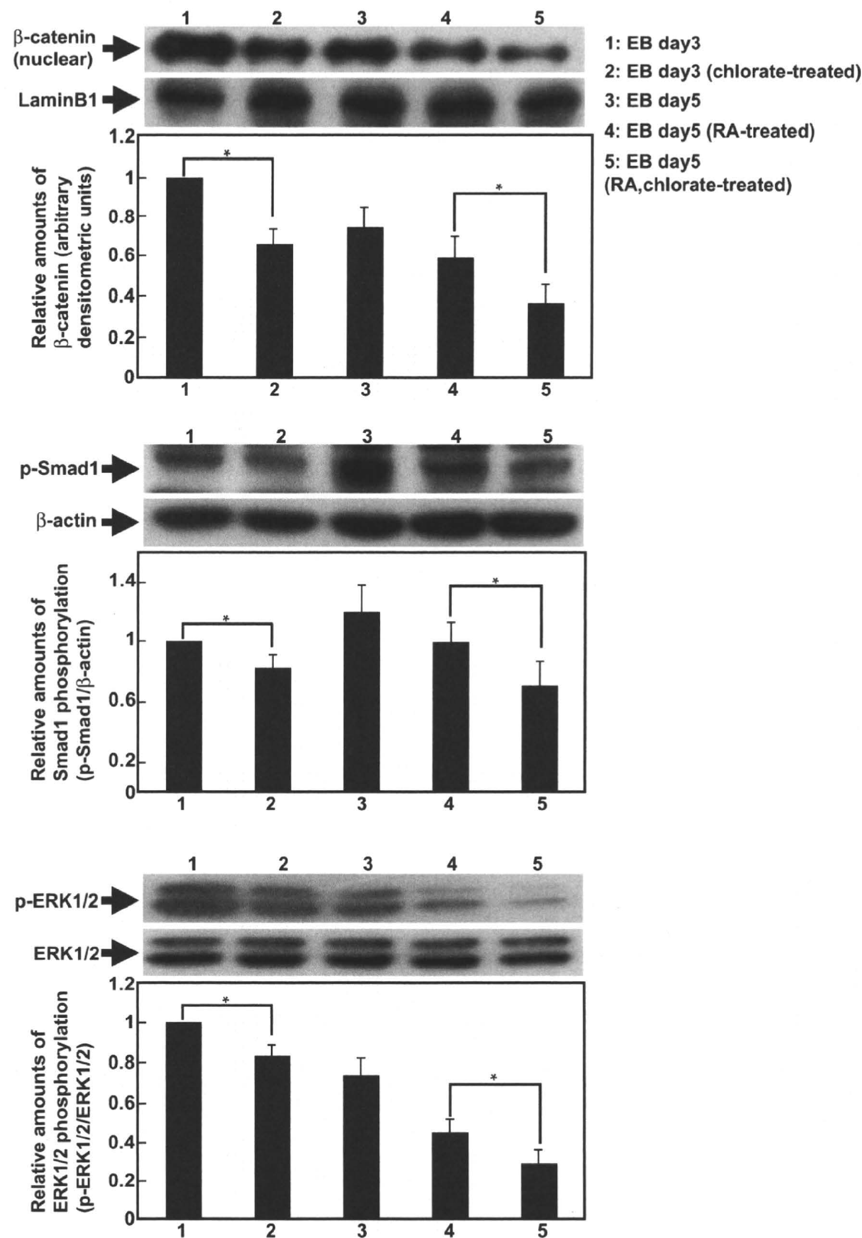

Fig. 3. Signaling via several pathways is decreased in chlorate-treated EBs. Western blotting of downstream signaling components 3 or 5 days after EB formation. Representative immunoblots from two independent experiments are shown. The histograms show mean densitometric readings ± SD of β-catenin or the phosphorylated proteins/loading controls after normalization against the values obtained with untreated EBs cultured for 3 days (value = 1). Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *$P < 0.05$.

3. Results and discussion

Firstly, we examined whether sulfation of glycans in EBs was reduced by chlorate treatment. HS and CS are functionally important sulfated glycans that are expressed until at least 8 days after EB formation in mESCs [13]. Thus, we focused on HS and CS in this study. Three days after EB formation, FACS analysis of chlorate-treated EBs revealed poor staining by the anti-HS and the anti-CS antibodies, indicating that sulfation on HS and CS was reduced (Fig. 1).

Signaling by both Wnt and BMP is essential for the choice of lineage between mesoderm and ectoderm: the reduction of both Wnt and BMP signaling inhibits mesodermal differentiation and enhances ectodermal differentiation [22,23]. Thus, we assumed that a reduction in Wnt and BMP signaling at the point when mesoderm starts to differentiate (2–4 days after EB formation) would accelerate neural differentiation. Therefore, we examined EB differentiation in response to chlorate treatment 2 days after EB formation. Real-time PCR analysis at 0–4 days after EB formation showed that the expression of early mesoderm markers (*Brachyury*, *Goosecoid*) did not increase in a time-dependent manner in chlorate-treated EBs, although it did in untreated EBs (Fig. 2A). In contrast, expression of ectoderm markers (*Mash1*, *Pax6*) was higher in chlorate-treated EBs than in untreated EBs at 4 days after EB formation (Fig. 2A). These results indicate that chlorate treatment from 2 days after EB formation affected lineage choice between mesoderm and ectoderm: it inhibited mesodermal differentiation but induced ectodermal differentiation. As shown in

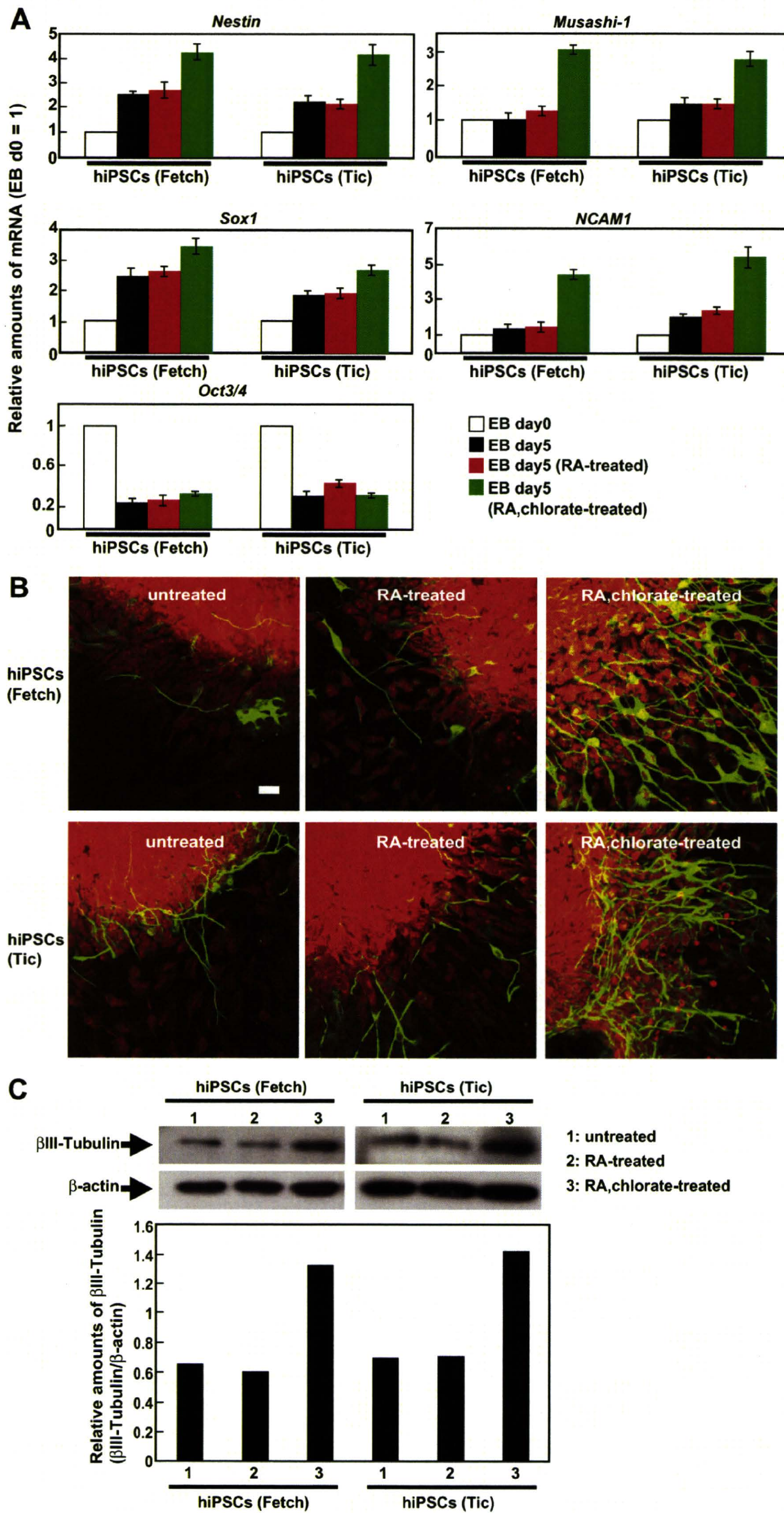

Fig. 4. Reduction of sulfation accelerates neural differentiation of hiPSCs. (A) Real-time PCR analysis of neural markers (*Nestin*, *Musashi-1*, *NCAM1* and *Sox1*) and an undifferentiated cell marker (*Oct3/4*) 5 days after EB formation. The relative amounts of each mRNA were normalized by *GAPDH* mRNA. The results are shown after normalization against the values obtained with undifferentiated hiPSCs (value = 1). The values shown are the means ± SD of duplicate measurements from representative experiments. Two independent experiments were performed. (B) Immunocytochemical staining 2 days after replating of EBs. Representative confocal images from two independent experiments are shown (βIII-tubulin, green; PI, red). Scale bar, 20 μm. (C) Western blotting 2 days after replating of EBs. Representative immunoblots from two independent experiments are shown. The histogram shows mean densitometric readings ± SD of βIII-tubulin/loading controls.

Fig. 3, Wnt/β-catenin, BMP/Smad, and FGF/ERK signaling, which are involved in the mesodermal differentiation of mESCs [24], were reduced in chlorate-treated EBs. Therefore, the inhibition of these signaling pathways by chlorate treatment seemed to cause defects in mesodermal differentiation and induced ectodermal differentiation. Chlorate treatment from the start of EB formation inhibited initial differentiation, as reported previously (data not shown, [25]). Thus, chlorate treatment from the appropriate time induces differentiation into a specific lineage, in particular neural differentiation.

Next, we investigated further the neural differentiation of chlorate-treated EBs. Firstly, we examined the expression of neural differentiation markers (Fig. 2B). Even at 5 days after EB formation, the expression levels of these markers were significantly higher in chlorate-treated EBs than in EBs only treated with RA, which indicated that differentiation into neural stem/neural progenitor cells and neural precursor cells was accelerated by chlorate treatment. These results were thought to be due to the reduction of Wnt and BMP signaling as shown in Fig. 3. Because, signaling by BMP and Wnt inhibits the neurogenesis of mESCs via EB formation [26,27]. Secondly, we examined the ability of chlorate-treated EBs to differentiate into mature neurons. Immunocytochemical staining for the mature neuron marker βIII-tubulin showed that only 2 days after plating, chlorate-treated EBs generated extremely dense networks of neurite outgrowths, which were not seen with EBs only treated with RA (Fig. 2C). We confirmed by western blotting that the level of βIII-tubulin in chlorate-treated cells was significantly higher than that in cells only treated with RA (Fig. 2D). Taken together, these results demonstrated that the reduction of sulfation by chlorate treatment accelerated the neurogenesis of mESCs.

As described above, the reduction of sulfation on HS and CS by chlorate treatment (Fig. 1) caused defects in Wnt/β-catenin, BMP/Smad, and FGF/ERK signaling during EB formation (Fig. 3), which was followed by the acceleration of neural differentiation (Fig. 2). These results are consistent with previous report [13], in which reduction of PAPST-dependent sulfation promotes neurogenesis of mESCs due to the decreased sulfation on HS and CS.

To date, several signaling pathways have been shown to contribute to the neural differentiation of hESCs and hiPSCs [18,28]. The BMP inhibitor Noggin and the inhibitor of the Lefty/Activin/TGFβ pathways SB431542 enhance neural differentiation [18]. Thus, we examined whether the inhibition of signaling pathways by chlorate treatment also enhanced the neural differentiation of hiPSCs. Real-time PCR analysis 5 days after EB formation showed that the expression of several neural stem/progenitor cell markers was higher in chlorate-treated EBs than in EBs only treated with RA, whereas expression of the marker of undifferentiated cells, *Oct3/4*, was reduced equally in both types of EBs (Fig. 4A). These results indicate that chlorate treatment accelerated the neural differentiation of hiPSCs. Furthermore, as shown in Fig. 4B and C, differentiation into mature neurons was induced markedly in chlorate-treated EBs.

In conclusion, we have demonstrated that the down-regulation of sulfation by chlorate treatment could accelerate neural differentiation of hiPSCs as well as mESCs. This acceleration was induced by the addition of chlorate at the correct time, and involved the inhibition of signaling pathways involved in the induction of mesodermal differentiation (Wnt, BMP, and FGF) and inhibition of neural differentiation (Wnt and BMP). This study as well as our previous report [13] have demonstrated that reduction of sulfation is effective to induce neural differentiation compared with the standard method using RA. In our previous report [13], we used RNAi method using retrovirus vector for long-term neural differentiation. This method requires great care and may give damages to the transfected cells. Moreover, the reduction of sulfation is more drastic in chlorate-treated cells than in *PAPST*-knockdown cells. This reflects more rapid induction of neural differentiation in chlorate-treated cells as shown in this report than in *PAPST*-knockdown cells. Furthermore, in hiPSCs including hESCs, efficiency of gene transfer is very low and for clinical application gene transfer should be avoided. Therefore, chlorate is useful for rapid, simple, and safety reduction of sulfation for rapid neural differentiation of hiPSCs, possibly including hESCs. Taken together, we propose that chlorate, which is available at low cost, could be used to achieve efficient and rapid neural induction of hiPSCs as well as mESCs in place of expensive signaling inhibitors.

Acknowledgments

Our research was partially supported by funds from Ministry of Education, Culture, Sports, Science and Technology (MEXT), the Grant-in-Aid for Scientific Research (B) to SN, 20370051, 2008–2010, and from MEXT, the Matching Fund for Private Universities, S0901015, 2009–2014.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.085.

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Biomed Microdevices (2010) 12:1097–1105
DOI 10.1007/s10544-010-9464-8

Enhanced effects of secreted soluble factor preserve better pluripotent state of embryonic stem cell culture in a membrane-based compartmentalized micro-bioreactor

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Published online: 1 September 2010
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Abstract Pluripotent stem cells are under the influence of soluble factors in a diffusion dominant *in vivo* microenvironment. In order to investigate the effects of secreted soluble factors on embryonic stem cell (ESC) behavior in a diffusion dominant microenvironment, we cultured mouse ESCs (mESCs) in a membrane-based two-chambered micro-bioreactor (MB). To avoid disturbing the cellular environment in the top chamber of the MB, only the culture medium of the bottom chamber was exchanged. Cell growth in the MB after 5 days of culture was similar to that in conventional 6-well plate (6-WP) and membrane-based Transwell insert (TW) cultures, indicating adequate nutrient supply in the MB. However, the cells retained higher expression of pluripotency markers (Oct4, Sox2 and Rex1) and secreted soluble factors (FGF4 and BMP4) in the MB. Inhibition of FGF4 activity in the MB and TW resulted in a similar cellular response. However, in contrast to the TW, inhibition of BMP4 activity revealed that autocrine action of the upregulated BMP4, which acted cooperatively with leukemia inhibitory factor (LIF), upregulated the pluripotency markers expression in the MB culture. We propose that BMP4 accumulated in the diffusion dominant microenvironment of the MB upregulated its own expression by a positive feedback mechanism—in contrast to the macro-scale culture systems—thereby enhancing the pluripotency of mESCs. The micro-scale culture platform developed in this study enables the investigation of the effects of soluble factors on ESCs in a diffusion dominant microenvironment, and is expected to be used to modulate the ESC fate choices.

Keywords Embryonic stem cell · Soluble factors · Diffusion · Microenvironment · Micro-bioreactor

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1 Introduction

The autocrine and paracrine actions of soluble factors have an important role in directing pluripotent stem cell fate choices *in vivo* (Gadue et al. 2005; Loebel et al. 2003). Pluripotent stem cells and their progenies remain in a diffusion dominant microenvironment enclosed by the trophectoderm and extra-embryonic part until an appreciable amount of mass flow by convection occurs after the onset of blood circulation (Nagy et al. 2003). At the initial stage of embryo development, the fate of pluripotent cells is influenced by the adequate signaling

of soluble factors in the microenvironment. *In vitro*, ESC fate is also modulated by soluble factors (Kunath et al. 2007; Ying et al. 2003a). Although exogenous soluble factors can be added to the *in vitro* culture systems to control ESC fate, it is necessary to consider the influence of endogenous soluble factors which are secreted by the cells (Wiles and Proetzel 2006). This is highlighted by the fact that the addition of exogenous soluble factors has little influence on the initial differentiation of embryoid bodies (EBs) but influences the successive maturation of differentiated progenies towards the matured cell types (Ogawa et al. 2005; Wiles and Proetzel 2006). Furthermore, neuronal stem cells can be derived efficiently from mouse ESCs (mESCs) without the addition of exogenous factors (Ying et al. 2003b). Therefore, a culture system which mimics the diffusion dominant nature of the *in vivo* microenvironment is of great importance in order to improve our understanding of stem cell biology and control the stem cell fate decision (Loebel et al. 2003; Murry and Keller 2008).

Microfluidic technology provides advanced tools to develop micro-scale culture systems in an *in vivo* relevant dimension as well as to control mass transfer modes in the cellular microenvironment (Meyvantsson and Beebe 2008). Various micro-scale culture systems have been developed for ESC culture, but little is known about the effects of secreted soluble factors in these systems. Moreover, before proceeding to the differentiation of ESCs, it is necessary to characterize the differences between the micro and macro-scale cultures, namely regarding the effects of cell secreted soluble factors on ESC behavior. Human ESCs (hESCs) cells were cultured in straight micro-channels in static (Abhyankar et al. 2003) and semi-static (Korin et al. 2009) conditions. Although these cultures facilitated the accumulation of soluble factors around the cells owing to the diffusion dominant nature of static micro-scale culture, their effects on the cells were not investigated. Furthermore, the environment changed abruptly because of the daily replacement of the total culture medium. In micro-fabricated wells, hESCs were found to remain undifferentiated for more than two weeks (Mohr et al. 2006). The reason for that was not identified, but most likely resulted from soluble factors, cell-cell contacts and the extra-cellular matrix (ECM) produced by the cells. Some studies focused on controlling ESC microenvironment using perfusion-based systems (Figallo et al. 2007; Kim et al. 2006). In one of those studies, mESCs were cultured in microfluidic arrays at different flow rates, and the cell colonies showed flow-dependent size variations (Kim et al. 2006). This was attributed to the amount of nutrient delivery as well as the removal of waste and secreted factors. Although perfusion is a way to supply enough nutrients to cells for long-term culture and control the cellular microenvironment by removal of the secreted soluble factors, it disturbs the cellular diffusion-based microenvironment (Walker et al. 2004).

In this context, we developed a membrane-based two-chambered micro-bioreactor (designated as MB hereafter) and culture conditions for ESCs to investigate the influence of secreted soluble factors on cells by mimicking the diffusion-dominant *in vivo* microenvironment. The culture medium of the top chamber was not replaced during the culture period to avoid disturbance in the cellular microenvironment. In contrast, the culture medium of the bottom chamber was exchanged daily to maintain a sufficient nutrient supply. We cultured mESCs for five days in leukemia inhibitory factor (LIF) supplemented culture medium to study the effects of soluble factors on cellular behavior, such as cell-cell interactions, cell proliferation and differentiation, in which the influence of secreted soluble factors is important (Yu et al. 2005). In the LIF supplemented medium, BMP4 synergistically interacts with LIF to preserve the mESC pluripotency by resisting the differentiation inducing action of FGF4 (Ying et al. 2008c). Therefore, the cell states in the MB, membrane-based macro-scale Transwell Insert (TW) and conventional 6-well plate (6-WP) cultures were compared by the expression of pluripotency markers (Oct4, Sox2, Rex1 and Nanog) and cell secreted soluble factors (FGF4 and BMP4). In addition, we performed cell culture experiments by inhibiting signaling components of FGF4 and BMP4 in the MB and TW. Then, the gene expressions of inhibited and non-inhibited cultures were compared to discern the effects of soluble factors in the micro and macro-scale culture systems.

2 Materials and methods

2.1 Design of the MB

Figure 1 shows the design details of the MB. The reactor had two round chambers (top and bottom) with an area of 2.27 cm^2. They were kept separated by a porous membrane. Each of the chambers' height and volume were 500 μm and 114 μL, respectively. The chambers contained 13 pillars (1 mm in diameter) that kept the membrane horizontal, and enabled a more homogeneous cell seeding on the membrane. Cells were cultured on the top face of the membrane. To avoid culture area other than the membrane, two feeding holes in the top chamber were drilled at the chamber perimeter. On the other hand, feeding holes in the bottom chamber were made approximately 0.7 cm away from the chamber perimeter to get clearance from the membrane perimeter.