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Expression profiles of protein tyrosine kinase genes in human embryonic stem cells

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Abstract

Complex signaling pathways operate in human embryonic stem cells (hESCs) and are coordinated to maintain self-renewal and stem cell characteristics in them. Protein tyrosine kinases (PTKs) participate in diverse signaling pathways in various types of cells. Because of their functions as key molecules in various cellular processes, PTKs are anticipated to have important roles also in hESCs. In this study, we investigated the roles of PTKs in undifferentiated and differentiated hESCs. To establish comprehensive PTK expression profiles in hESCs, we performed reverse transcriptase PCR using degenerate primers according to the conserved catalytic PTK motifs in both undifferentiated and differentiated hESCs. Here, we identified 42 different kinases in two hESC lines, including 5 non-receptor tyrosine kinases (RTKs), 24 RTKs, and 13 dual and other kinases, and compared the protein kinase expression profiles of hESCs and retinoic acid-treated hESCs. Significantly, up- and downregulated kinases in undifferentiated hESCs were confirmed by real-time PCR and western blotting. *MAP3K3*, *ERBB2*, *FGFR4*, and *EPHB2* were predominantly upregulated, while *CSF1R*, *TYRO3*, *SRC*, and *GSK3A* were consistently downregulated in two hESC lines. Western blot analysis showed that the transcriptional levels of these kinases were consistent with the translational levels. The obstruction of upregulated kinases' activities using specific inhibitors disturbed the undifferentiated status and induced the differentiation of hESCs. Our results support the dynamic expression of PTKs during hESC maintenance and suggest that specific PTKs that are consistently up- and downregulated play important roles in the maintenance of stemness and the direction of differentiation of hESCs.

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Introduction

Human embryonic stem cells (hESCs) have the ability to self-renew and differentiate into a variety of cell types within the three embryonic germ layers (Thomson *et al*. 1998). To identify the key molecules involved in maintaining stemness of hESCs, transcriptome characterization procedures, such as microarray (Ramalho-Santos *et al*. 2002, Sato *et al*. 2003, Sperger *et al*. 2003, Dvash *et al*. 2004), serial analysis of gene expression (Richards *et al*. 2004), and expressed sequence tags (ESTs; Brandenberger *et al*. 2004), have been employed. Molecules participating in several signaling pathways, including fibroblast growth factor (FGF), WNT, NODAL, PI3K/AKT, MAP kinase (MAPK)/ERK, and NF-κB, may be involved in maintaining the stemness of hESCs (Sato *et al*. 2003, Brandenberger *et al*. 2004, Armstrong *et al*. 2006, Rho *et al*. 2006).

Generally, protein tyrosine kinases (PTKs) play pivotal roles in cell proliferation, apoptosis, oncogenesis, differentiation, and development (Hunter 1987). These proteins are among the key molecules that regulate signaling pathways, but only account for less than 10% of the total cellular kinases (Hunter 1994). PTKs are classified into receptor tyrosine kinases (RTK) and cytoplasmic non-RTKs (NRTK). In humans, 90 PTKs have been identified to date, comprising 58 receptor and 32 NRTKs (Robinson *et al*. 2000). Expression of most PTKs may be tightly regulated to retain unique features of a specific cell type. However, limited information is available on the molecular behavior of PTKs in hESCs.

Here, we present the PTK-focused expression profiles of hESCs. The transcriptional profiles of protein kinases (PKs) from hESCs are compared with those of retinoic acid (RA)-treated hESCs by an RT-PCR-based cloning technique using degenerate primers corresponding to the highly conserved catalytic domains of PTKs. We analyzed 1298 clones and identified 42 kinase genes in two genetically independent hESC lines. Our PTK profiling data should be valuable in understanding the

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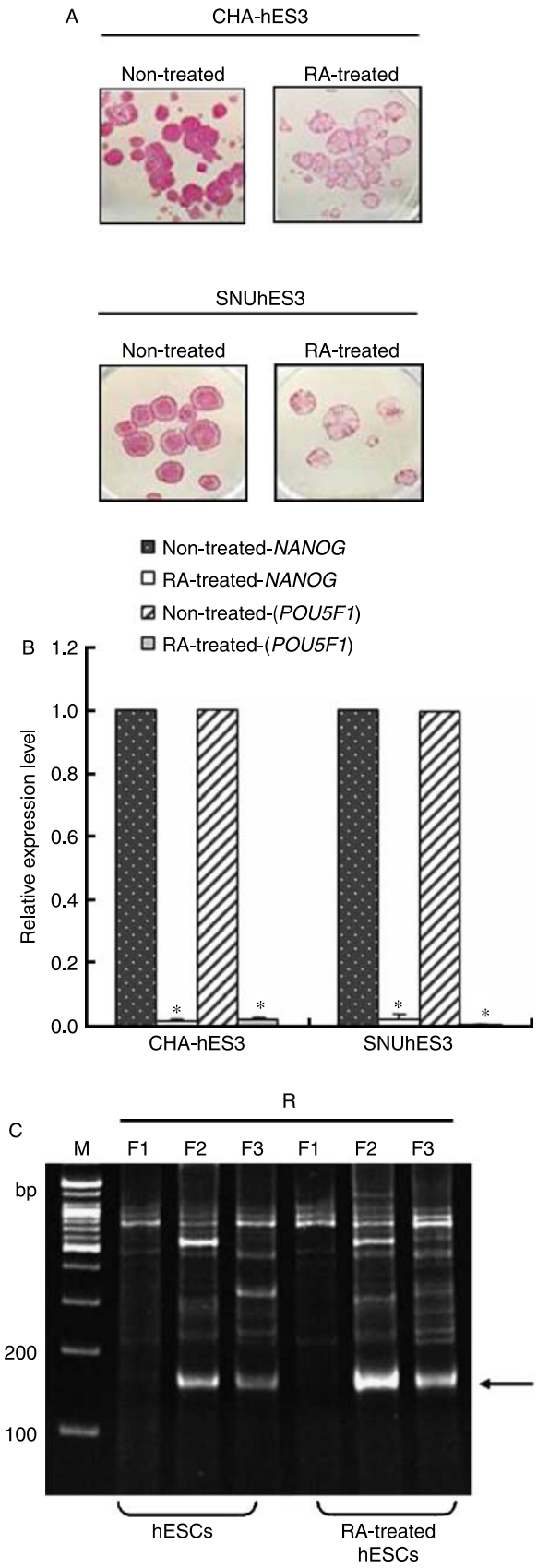

molecular mechanisms underlying the stemness, developing the novel biomarkers for the pluripotency, and identifying the potential regulators for differentiation in hESCs.

Results

Identification of the tyrosine kinase family in hESCs

To elucidate the molecular mechanisms of PTKs regulating the stemness of hESCs, differentiated cells were derived from the hESCs used as controls. To induce differentiation, hESCs were treated with RA for 5 days. As shown in Fig. 1A, alkaline phosphatase (AP) activity was considerably weak in RA-treated hESCs, compared with control hESCs. Moreover, transcriptional expression of stem cell marker genes, such as *POU5F1 (OCT4)* and *NANOG*, was remarkably reduced as assessed by real-time PCR (Fig. 1B). To assess the degree of diversity in differentiation capacity, we performed the real-time PCR analyses of the lineage markers for endoderm (amylase, albumin), mesoderm (*GATA2*), and ectoderm (*NCAM*). The RA-treated hESCs used in this study expressed diverse lineage markers (Supplementary Fig. 1, which can be viewed online at www.reproduction-online.org/supplemental). Thus, the RA treatment appeared to result in the loss of stemness with no preference for differentiation towards a specific lineage, consistent with a previous report (Draper *et al.* 2002). To determine the transcriptional patterns of PTKs, degenerate RT-PCR was performed in both control and RA-treated hESCs. Degenerate primers were designed on the basis of conserved motifs within the catalytic domains of tyrosine kinase, DFG and DVW. The DFG motif is present in all PK types; while DVW is primarily encoded by tyrosine kinases, but a small subset of serine/threonine kinases also contain the motif (Robinson *et al.* 1996, Lin *et al.* 1998). In this study, the forward (F1, F2, and F3) and reverse primer (R) are based on DFG and DVW motifs respectively (Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental). DNA fragments of 150–170 bp were successfully amplified by degenerate PCR (Fig. 1C). The F1-R combination yielded the least PCR products in two hESC lines. Similar results have been observed with other tissues and cell lines (Lin *et al.* 1998, Wu *et al.* 2000). We performed two independent degenerate PCRs for each hESC lines and pooled the amplification products together respectively,

Figure 1 Induction of hESC differentiation by retinoic acid (RA). (A) Alkaline phosphatase staining of hESC colonies. (B) Expression of hESC markers, *POU5F1* and *NANOG*, by real-time PCR analysis. Statistical significance of data was assessed using Student's *t*-test (*$P<0.01$). (C) Representative gel electrophoresis of amplified protein tyrosine kinase RT-PCR products (150–170 bp). RT-PCR using a combination of degenerate primers, F1, F2, F3, and R, was performed, as described in the Materials and Methods section.

for subsequent analysis. The pooled amplified products were eluted, cloned, and sequenced. BLASTN analyses revealed that 90.0% (743/826 clones) and 94.3% (445/472 clones) genes from CHA-hES3 and SNUhES3 encoded human PKs respectively (Table 1). Thus, we presented a high fidelity procedure for identifying PKs in hESCs. Among the identified genes, there were 36 and 31 non-redundant human kinase genes in CHA-hES3 and SNUhES3 respectively. Non-PKs were additionally detected, possibly as a result of PCR-related mispriming. Murine PKs were observed at a low frequency in both hESC types, which appear to be derived from feeder cells. Twenty-three human PTK genes and two serine/threonine kinases were commonly expressed in the two hESCs (Table 2).

Differential expression of PK genes in hESCs

To identify the candidate genes associated with the stemness of hESCs, expression profiles of PK genes in hESCs were compared with those of RA-treated hESCs by analyzing gene frequency (Table 2). In total, 386 clones from CHA-hES3 cells and 357 clones from RA-treated CHA-hES3 cells were assessed. Out of the 36 PKs identified in CHA-hES3 cells, 15 were transcriptionally upregulated and 19 were downregulated, compared with RA-treated CHA-hES3 cells. Two PK genes were equally expressed. Additionally, 228 clones from SNUhES3 cells and 217 clones from RA-treated SNUhES3 cells were analyzed. We identified 31 different PK genes in SNUhES3 cells. Of these, 17 PKs were transcriptionally upregulated and 14 were downregulated. In total, 42 kinases (including 5 NRTKs, 24 RTKs, and 13 dual and other kinases) were identified in CHA-hES3 and SNUhES3 cells (Table 2). Out of the 42 genes, 11 (*MAP3K3*, *ERBB2*, *FGFR4*, *EPHB2*, *CLK3*, *DDR1*, *MAP2K4*, *FGFR1*, *FGFR3*, *KDR*, and *MAP3K11*) and 10 (*CSF1R*, *TYRO3*, *SRC*, *GSK3A*, *PDGFRB*, *PLK4*, *ABL1*, *EPHA2*, *EPHB3*, and *MERTK*) were commonly up- and downregulated in both hESC lines respectively. The cellular functions of the kinase genes identified in this study are presented in Table 3. These PK profiles obtained using gene frequency analyses provide valuable information on the dynamic behavior of these proteins between undifferentiated and differentiated hESCs.

Table 1 Identification of protein kinases (PKs) expressed in human embryonic stem cells (hESCs) by RT-PCR using degenerate primers.

hESC lines	Category	Clones	(%)	Genes
CHA-hES3	Human PKs[a]	743	(90.0)	36
	Murine PKs[a]	4	(0.5)	4
	Non-PKs	79	(9.6)	41
	Known[b]	56		21
	No hits[c]	23		20
	Total	826		81
SNUhES3	Human PKs[a]	445	(94.3)	31
	Murine PKs[a]	6	(1.3)	3
	Non-PKs	21	(4.4)	18
	Known[b]	17		14
	No hits[c]	4		4
	Total	472		52

[a]Protein tyrosine kinases, dual specific kinases, and some serine/threonine kinases are included. [b]Genes of known function that are not human PTK genes. [c]No significant similarity to any known genes by BLASTN.

Validation of selected tyrosine kinases by real-time PCR and western blotting

To validate the differential expression of PK genes between normal and differentiated hESCs, further experiments were performed on several selected genes. Prior to choosing the genes for ensuing analyses, we needed to set up selection criteria because the two different hESC lines of different genetic backgrounds that we analyzed showed some variations in gene expression patterns and possibly in preferential differentiation potential, as reported previously (Abeyta *et al.* 2004, Skottman *et al.* 2005). We selected the genes using the following criteria: (i) The expression of genes should be commonly up- and downregulated in both hESC lines respectively. (ii) Among commonly up- and downregulated genes, the significant *P* values of the genes from the gene frequency analysis on PK expression profile should be lower than 0.01 in at least one of the two hESC lines. Based on this, we found that four upregulated (*MAP3K3*, *ERBB2*, *FGFR4*, and *EPHB2*) and four downregulated (*CSF1R*, *TYRO3*, *SRC*, and *GSK3A*) genes fitted the criteria (Table 3). To verify more quantitatively, eight selected kinases were analyzed by real-time PCR. Four PTK genes (*MAP3K3*, *ERBB2*, *FGFR4*, and *EPHB2*) were highly expressed in both CHA-hES3 and SNUhES3 cells, compared with RA-treated hESCs (Fig. 2A), whereas four selected genes (*CSF1R*, *TYRO3*, *SRC*, and *GSK3A*) displayed lower transcriptional levels in both hESC lines, compared with RA-treated hESCs (Fig. 2B). Thus, the expression patterns of kinase genes detected using gene frequency analysis were consistent with those determined from quantitative real-time PCR. It could be guessed that the differential expressions of the target kinase genes were observed uniquely from RA-directed differentiation but not from other general differentiation protocols. To test this possibility, we prepared embryoid bodies (EBs) from CHA-hES3, which are widely regarded as spontaneously differentiated cells representing a mixture of various cell types, and examined the target gene expression with them. The results of quantitative real-time PCR with EBs showed both up- and downregulated kinases to be similarly expressed respectively as the results with RA-treated samples (Supplementary Fig. 2, which can be viewed online at www.reproduction-online.org/supplemental). Thus, it might be assumed that expressions of eight selected kinases are not dependent on particular differentiation protocols.

Table 2 Expression profiles of protein kinases in human embryonic stem cells (hESCs).

Kinase	Amino acid sequence	Frequency (%)[a]					
		CHA-hES3	RA-treated CHA-hES3	PE[b]	SNUhES3	RA-treated SNUhES3	PE[a]
Non-receptor PTK							
ABL1	**DFG**LSRLMTGDT----YTAHAG-AKFPIK**W**TAP----**E**SLAYNK-----FSIKS--**DVW**	18.7	21.3	−	7.9	16.1	−
FES	**DFG**MSREEADGV----YAASGGLRQVPVK**W**TAP----**E**ALNYGR-----YSSES--**DVW**	0.8	2.0	−	2.6	0.5	+
JAK3	**DFG**LAKLLPLDKDY---YVVREPGQSPIF**W**YAP----**E**SLSDNI-----FSRQS--**DVW**	0.3	0.0	+	0.0	0.0	NF
SRC	**DFG**LARLIEDNE----YTARQG-AKFPIK**W**TAP----**E**AALYGR-----FTIKS--**DVW**	0.8	3.6	−	0.4	1.4	−
TYK2	**DFG**LAKAVPEGHEY---YRVREDGDSPVF**W**YAP----**E**CLKEYK-----FYYAS--**DVW**	0.3	0.0	+	0.0	0.0	NF
Receptor PTK							
CSF1R	**DFG**LARDIMNDSNY----IVKGNARLPVK**W**MAP----**E**SIFDCV-----YTVQS--**DVW**	0.0	4.5	−	0.0	6.0	−
DDR1	**DFG**MSRNLYAGDYY----RVQGRAVLPIR**W**MAW----**E**CILMGK-----FTTAS--**DVW**	1.0	0.0	+	1.3	0.5	+
EPHA1	**DFG**LTRLL--DDFDGTYETQ-G-GKIPIR**W**TAP----**E**AIAHRI-----FTTAS--**DVW**	0.3	0.3	•	0.0	0.0	NF
EPHA2	**DFG**LSRVLEDDP-EATYTTS-G-GKIPIR**W**TAP----**E**AISYRK-----FTSAS--**DVW**	0.0	0.3	−	2.2	2.3	−
EPHA4	**DFG**MSRVLEDDP-EAAYTTR-G-GKIPIR**W**TAP----**E**AIAYRK-----FTSAS--**DVW**	0.0	0.0	NF	0.0	0.5	−
EPHB1	**DFG**LSRYLQDDTSDPTYTSSLG-GKIPVR**W**TAP----**E**AIAYRK-----FTSAS--**DVW**	1.6	0.0	+	0.4	0.5	−
EPHB2	**DFG**LSRFLEDDTSDPTYTSALG-GKIPIR**W**TAP----**E**AIQYRK-----FTSAS--**DVW**	29.5	15.1	+	43.9	43.3	+
EPHB3	**DFG**LSRFLEDDPSDPTYTSSLG-GKIPIR**W**TAP----**E**AIAYRK-----FTSAS--**DVW**	0.0	0.8	−	0.0	0.5	−
EPHB4	**DFG**LSRFLEENSSDPTYTSSLG-GKIPIR**W**TAP----**E**AIAFRK-----FTSAS--**DAW**	0.0	0.3	−	0.0	0.0	NF
ERBB1	**DFG**LAKLLGAEEKE---YHAEG-GKVPIK**W**MAL----**E**SILHRI-----YTHQS--**DVW**	0.0	0.8	−	0.0	0.0	NF
ERBB2	**DFG**LARLLDIDETE---YHADG-GKVPIK**W**MAL----**E**SILRRR-----FTHQS--**DVW**	17.1	7.3	+	11.0	5.1	+
ERBB3	**DFG**VADLLPPDDKQ---LLYSE-AKTPIK**W**MAL----**E**SIHFGK-----YTHQS--**DVW**	0.0	0.3	−	0.0	0.0	NF
FGFR1	**DFG**LARDIHHIDYY----KKTTNGRLPVK**W**MAP----**E**ALFDRI-----YTHQS--**DVW**	6.5	5.0	+	2.6	0.9	+
FGFR3	**DFG**LARDVHNLDYY----KKTTNGRLPVK**W**MAP----**E**ALFDRV-----YTHQS--**DVW**	1.0	0.6	+	0.4	0.0	+
FGFR4	**DFG**LARGVHHIDYY----KKTSNGRLPVK**W**MAP----**E**ALFDRV-----YTHQS--**DVW**	3.4	1.1	+	4.4	0.0	+
FGFRL1	**DFG**GTTSFQCKVRS--DVKPVIQWLKRVEYGAEGRHNSTIDVGGQ---KFVVLPTG**DVW**	0.3	0.3	•	0.0	0.0	NF
FLT4	**DFG**LARDIYKDPDY----VRKGSARLPLK**W**MAP----**E**SIFDKV-----YTTQS--**DVW**	0.0	0.0	NF	0.4	0.0	+
INSRR	**DFG**MTRDVYETDYY----RKGGKGLLPVR**W**MAP----**E**SLKDGI-----FTTHS--**DVW**	0.5	0.6	−	0.9	0.0	+
KDR	**DFG**LARDIYKDPDY----VRKGDARLPLK**W**MAP----**E**TIFDRV-----YTIQS--**DVW**	0.5	0.0	+	0.4	0.0	+
MERTK	**DFG**LSKKIYSGDYY----RQGRIAKMPVK**W**IAI----**E**SLADRV-----YTSKS--**DVW**	0.0	0.3	−	0.0	0.5	−
MST1R	**DFG**LARDILDREYYS--VQQHRHARLPVK**W**MAL----**E**SLQTYR-----FTTKS--**DVW**	0.0	0.3	−	0.0	0.0	NF
PDGFRA	**DFG**LARDIMHDSNY----VSKGSTFLPVK**W**MAP----**E**SIFDNL-----YTTLS--**DVW**	0.0	0.0	NF	0.0	0.5	−
PDGFRB	**DFG**LARDIMRDSNY----ISKGSTFLPLK**W**MAP----**E**SIFNSL-----YTTLS--**DVW**	0.3	2.5	−	0.0	0.9	−
TYRO3	**DFG**LSRKIYSGDYY----RQGCASKLPVK**W**LAL----**E**SLADNL-----YTVQS--**DVW**	1.8	14.8	−	2.2	11.5	−
Dual & others							
ARAF	**DFG**LATVKTRWSGA----QPLEQPSGSVL**W**MAA----**E**VIRMQDP--NPYSFQS--**DVY**	0.0	0.0	NF	0.4	0.0	+
CAMKK2	**DFG**VSNEFK---GS---DALLSNTVGTPAFMAP----**E**SLSETRK--IFSGKAL--**DVW**	0.0	0.0	NF	0.4	0.0	+
CDK4	**DFG**LARIYS----Y---QMALTPVVVTLW**Y**RAP----**E**VLLQST-----YATPV--**DMW**	0.0	0.0	NF	0.0	0.5	−
CLK1	**DFG**SA-TYD----D---EH-HSTLVSTRH**Y**RAP----**E**VILALG-----WSQPC--**DVW**	0.0	0.6	−	0.0	0.0	NF
CLK3	**DFG**SA-TFD----H---EH-HTTIVATRH**Y**RPP----**E**VILELG-----WAQPC--**DVW**	1.6	0.6	+	3.5	0.9	+
GSK3A	**DFG**SAKQLV----R---GEPNVSYICSRY**Y**RAP----**E**LIFG----ATDYTSSI--**DVW**	2.8	11.2	−	2.2	4.6	−
ICK	**DFG**LAREIR----S---KPPYTDYVSTRW**Y**RAP----**E**VLLRSTN----YSSPI--**DVW**	0.0	0.6	−	0.0	0.0	NF
MAP2K3	**DFG**ISGYLV----D---SVAKTMDAGCKP**Y**MAP----**E**RINP-ELNQKGYNVKS--**DVW**	1.3	0.0	+	0.0	0.0	NF
MAP2K4	**DFG**ISGQLV----D---SIAKTRDAGCRP**Y**MAP----**E**RIDP-SASRQGYDVRS--**DVW**	1.6	0.3	+	1.8	0.5	+
MAP2K7	**DFG**ISGRLV----D---SKAKTRSAGCAA**Y**MAP----**E**RIDPPDPTKPDYDIRA--**DVW**	0.0	0.3	−	0.4	0.0	+
MAP3K3	**DFG**ASKRLQTICMS---GTGMRSVTGTPY**W**MSP----**E**VISGEG-----YGRKA--**DVW**	6.5	1.4	+	9.6	0.5	+
MAP3K11	**DFG**LAREWHKTTQM----SAAG----TYA**W**MAP----**E**VIKAST-----FSKGS--**DVW**	1.3	0.8	+	0.4	0.0	+
PLK4	**DFG**LATQLK---MP---HEKHYTLCGTPN**Y**ISP----**E**IATRSA-----HGLES--**DVW**	0.5	2.2	−	0.0	2.3	−

[a]The frequency of each kinase was evaluated by dividing the number of clones of a kinase gene by the number of total clones of human origin from each sample. [b]PE indicates the patterns of expression of a gene in hESC as follows; +, upregulated; −, downregulated; •, equally expressed; NF, not found.

Table 3 Up- and downregulated kinase genes in human embryonic stem cells (hESCs).

Gene	**Gene description** (Gene title)	**Gene function**[b]	***P* value**[a]	
			CHA3-hES3	SNU3-hES3
Upregulated				
MAP3K3	MAP kinase kinase kinase 3	MAPKKK cascade	$P<0.001$	$P<0.001$
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	Cell proliferation	$P<0.001$	$P<0.03$
FGFR4	Fibroblast growth factor receptor 4	Cell communication	$P<0.05$	$P<0.002$
EPHB2	EPH receptor B2	Nervous system development	$P<0.001$	
CLK3	CDC-like kinase 3	Metabolism		$P<0.08$
DDR1	Discoidin domain receptor family, member 1	Cell adhesion	$P<0.08$	
MAP2K4	MAP kinase kinase 4	JNK cascade	$P<0.09$	
FGFR1	Fibroblast growth factor receptor 1	Cell growth, skeletal development		
FGFR3	Fibroblast growth factor receptor 3	Cell growth, skeletal development		
KDR	Kinase insert domain receptor	Cell fate commitment		
MAP3K11	MAP kinase kinase kinase 11	Cell proliferation, regulation of JNK cascade		
Downregulated				
CSF1R	Colony-stimulating factor 1 receptor	Cell proliferation	$P<0.001$	$P<0.001$
TYRO3	TYRO3 protein tyrosine kinase	Cell adhesion	$P<0.001$	$P<0.001$
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog	Cell communication	$P<0.001$	
GSK3A	Glycogen synthase kinase 3α	Metabolism	$P<0.001$	$P<0.1$
PDGFRB	Platelet-derived growth factor receptor, β-polypeptide	Cell communication	$P<0.02$	
PLK4	Polo-like kinase 4	Regulation of progression through cell cycle	$P<0.05$	$P<0.03$
ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1	Intracellular signaling cascade, cell adhesion		
EPHA2	EPH receptor A2	Neuron differentiation		
EPHB3	EPH receptor B3	Axon guidance		
MERTK	c-mer proto-oncogene tyrosine kinase	Cell surface receptor linked signal transduction		

[a]Probability of differential expression for each gene is calculated as described in the Materials and Methods section. [b]Gene function was analyzed using GO database.

We further examined the expressions of eight selected kinases at protein level in CHA-hES3 cells. The stemness marker, POU5F1, expression was markedly decreased in RA-treated CHA-hES3 cells. Undifferentiated hESCs expressed higher levels of MAP3K3, ERBB2, FGFR4, and EPHB2. On the other hand, CSF1R, TYRO3, SRC, and GSK3A are abundant in RA-treated CHA-hES3 cells. Therefore, the transcriptional profiles of eight PKs were consistent with the translational profiles in CHA-hES3 cells (Fig. 2C). It should be noted, however, that the protein level does not always correlate to the protein activity, which raises the need for measuring the level of the phosphorylated form of each kinase.

Effect of kinase inhibitors on the stemness of hESCs

Among the kinase genes consistently expressed in hESCs, it is possible that the highly expressed genes, such as *MAP3K3*, *ERBB2*, *FGFR4*, and *EPHB2*, play important roles in maintaining the undifferentiated hESC state. To further establish whether PTKs affect the stemness of hESCs, CHA-hES3 cells were separately exposed to commercially available PTK-specific inhibitors under the feeder-free system (Fig. 3). Following treatment with AG825 (an inhibitor of *ERBB2*) and PD173074 (an inhibitor of FGFR) for 3 days respectively, CHA-hES3 cells were morphologically differentiated (Fig. 3A) and showed weak AP activity (Fig. 3B) compared with the control group. *POU5F1* and *NANOG* transcripts were significantly reduced in inhibitor-treated hESCs (Fig. 3C). Flow cytometry analyses using antibody recognizing either AP or SSEA4 verified the result once again. While more than 90% of the control, CHA-hES3 cells had positive signals for the two markers, only 44.94 and 57.58% of AG825-treated cells and 34.09 and 26.64% of PD173074-treated cells were positive for AP and SSEA4 respectively (Fig. 3D). Our results imply that the inhibition of either *ERBB2* or *FGFR4* activity leads to loss of stemness in hESCs, supporting the theory that PTKs are involved in maintaining the undifferentiated state of hESC.

Discussion

The PTK profiling approach presented in this report has been used in several other research fields and helps to figure out the contributions of PTKs especially in tumor tissue and cancer cell line (Robinson *et al*. 1996, Lin *et al*. 1998). We adapted PTK profiling approach to hESC and rapidly isolated some PTKs that contribute to maintain undifferentiated status in hESCs. A few reports on PTKs that play key roles in controlling self-renewal

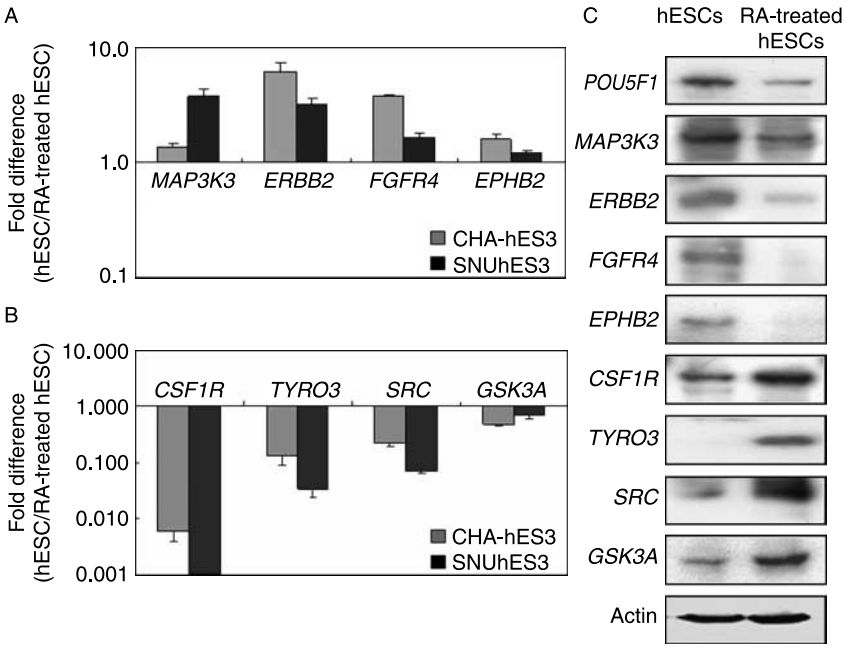

Figure 2 Verification of the up- and down-regulation of protein kinase (PK) genes in hESCs. Expression patterns of PK genes detected by gene frequency analysis were analyzed by real-time PCR. Four protein tyrosine kinase genes, *MAP3K3*, *ERBB2*, *FGFR4*, and *EPHB2*, were consistently upregulated in the two hESC lines, CHA-hES3 and SNU-hES3 (A), whereas four PK genes, *CSF1R*, *TYRO3*, *SRC*, and *GSK3A*, were consistently downregulated in two hESC lines (B). The expression levels of the PK genes in hESCs are compared, relative to levels in retinoic acid (RA)-treated hESCs, 1 representing unchanged expression and 0.1 signifying tenfold downregulation in hESCs. (C) Comparison of protein expression of selected kinases between undifferentiated hESC and differentiated hESCs. Twenty micrograms of each cell lysate from undifferentiated CHA-hES3 cells or RA-treated CHA-hES3 cells were processed for the western blotting analysis using specific antibodies. Actin was used as loading control.

and pluripotency in hESCs are available (Sato *et al*. 2003, Brandenberger *et al*. 2004, Skottman *et al*. 2005). These early studies demonstrate that FGF RTKs are related to the stemness of hESCs. In addition to FGF receptors (FGFRs), such as *FGFR4*, we also found that *MAP3K3*, *ERBB2*, *EPHB2*, *CSF1R*, *TYRO3*, *SRC*, and *GSK3A* were differentially expressed between undifferentiated and differentiated hESCs.

Among the PTKs described above, *MAP3K3*, *ERBB2*, *FGFR4*, and *EPHB2* were consistently upregulated in the undifferentiated state, but downregulated during differentiation (Fig. 2). Upregulation of these PTKs may be essential for maintaining the undifferentiated state of hESCs. FGFR is required to stimulate the phospholipase C γ, PI3K/AKT, and MAPK/ERK pathways (Eswarakumar *et al*. 2005). As observed from microarray (Sato *et al*. 2003, Skottman *et al*. 2005) and EST frequency analyses (Brandenberger *et al*. 2004), *FGF* receptors are highly expressed in hESCs. Our gene frequency analyses using degenerate PCR disclosed that three (FGFR1, FGFR3, and FGFR4) of the four FGFRs (FGFR1–4) were highly transcribed in hESCs, compared with differentiated hESCs (Table 2). Basic FGF2 (bFGF) is an essential factor in maintaining the undifferentiated state of hESCs through the FGFR1, FGFR3, and FGFR4 pathways (Thomson *et al*. 1998, Brandenberger *et al*. 2004). It is possible that since FGFR4 is consistently upregulated in two hESC lines, this receptor is associated with FGF2 and acts on subsequent signal transduction cascades. Together with FGFR4, we found *MAP3K3* and *ERBB2* also upregulated in undifferentiated hESCs. *MAP3K3* is a MAP kinase kinase kinase that activates the NF-κB and MAPK/ERK pathways (Zhang *et al*. 2006, Kim *et al*. 2007). *ERBB2*, a member of the epithermal growth factor (EGF) family of RTKs, plays important roles in cell proliferation, survival and differentiation (Hung & Lau 1999). *ERBB2* has no ligand-binding domain, but binds tightly to other ligand-bound EGF receptor family members to enhance kinase-mediated activation of downstream signaling pathways, such as PI3K/AKT and MAPK/ERK (Holbro & Hynes 2004). Microarray analysis revealed higher expression of *ERBB2* in control hESCs than differentiated tissue samples (Sperger *et al*. 2003). The PI3K/AKT, MAPK/ERK, and NF-κB signaling pathways are crucial for the maintenance of pluripotency in hESCs (Armstrong *et al*. 2006). Since *MAP3K3* and *ERBB2* function as active modulators in the PI3K/AKT, MAPK/ERK, and NF-κB signaling pathways, their upregulation is related to the stemness of hESCs. Interestingly, *MAP3K3* and *ERBB2* genes have binding sites for transcription factors, such as POU5F1, SOX2, and NANOG, upstream from the transcription initiation site (Boyer *et al*. 2005). Therefore, *MAP3K3* and *ERBB2* gene levels may be dependent on the expression of stem cell-specific factors, such as POU5F1, SOX2, and NANOG. In fact, the expression of *MAP3K3* and *ERBB2* was considerably decreased in differentiated hESCs (Fig. 2C). *EPHB2* RTK transcript levels were high in two hESC lines. EPH receptors, the largest known family of RTKs, are divided into two groups, based on the similarities in the extracellular domain sequences and affinities for binding ephrin-A and ephrin-B ligands, termed EphA and EphB respectively. The EPH/ephrin signaling pathway networks with the WNT signaling pathway during embryogenesis, tissue regeneration, and carcinogenesis (Katoh & Katoh 2006). The canonical

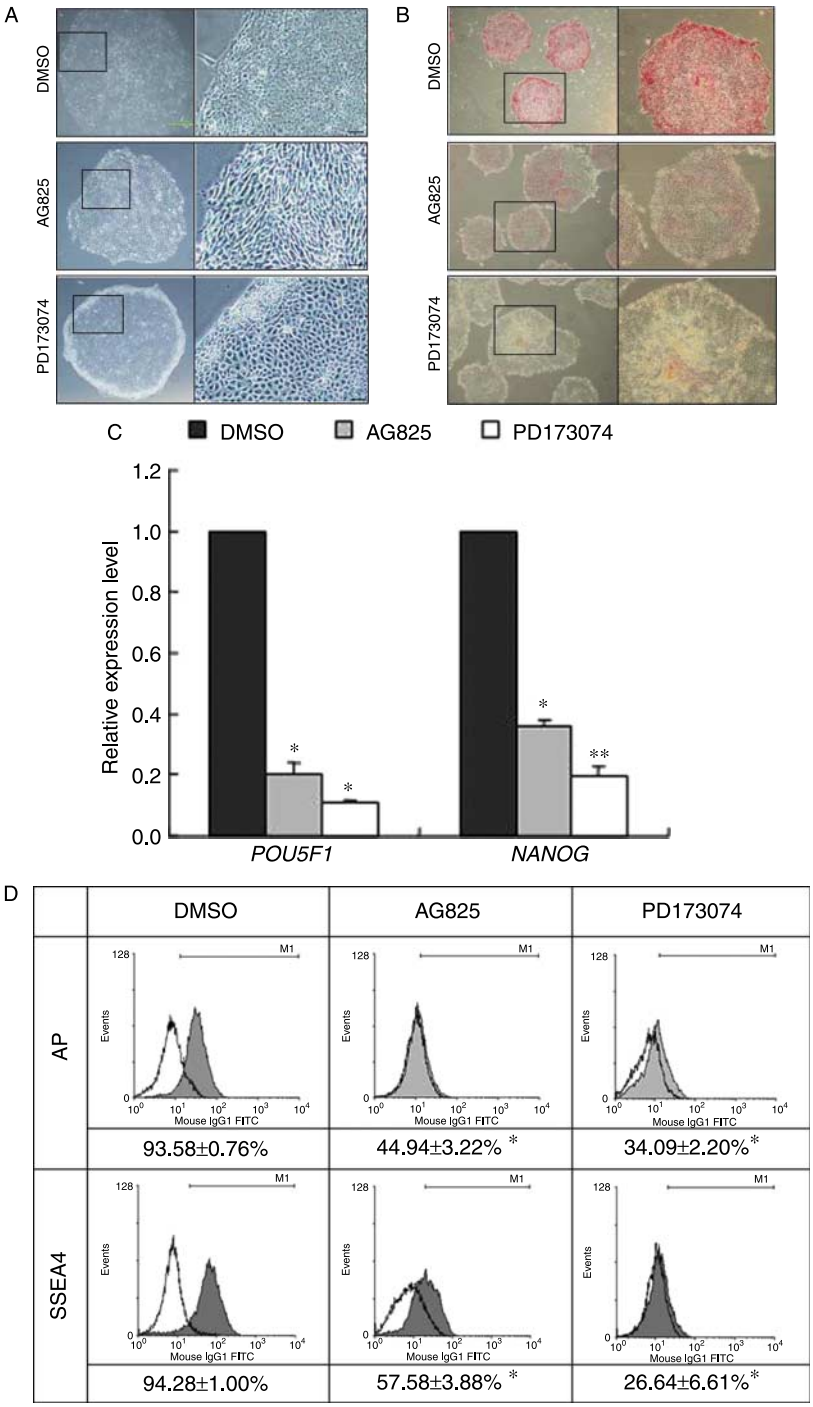

Figure 3 Effects of kinase inhibitors on the stemness in hESCs. CHA-hES3 cells were treated with inhibitors (10 μM for AG825 and 0.1 μM for PD173074) specific for ERBB2 and FGFR4 respectively. (A) Inhibitor-treated hESCs were altered in appearance and (B) exhibited indeterminate alkaline phosphatase (AP) staining. (C) Transcriptional expression of *POU5F1* and *NANOG* was significantly reduced in inhibitor-treated hESCs by real-time PCR analysis. The statistical significance of results was assessed using Student's *t*-test (*$P<0.05$, **$P<0.01$). (D) Flow cytometric analysis of cells labeled with antibody to AP or SSEA4. Shown are representative plots of three independent experiments. The values (mean ± S.E.M.) are the proportion of AP- or SSEA4-positive cells relative to total cells counted. Asterisks indicate that the values were significantly different from the value of DMSO control at $P<0.001$ level). Scale bars, 100 μm.

WNT signaling pathway has been reported to have a role in self-renewal of mouse and human ESCs (Sato *et al.* 2004). Therefore, the EPHB2 RTK may be involved in self-renewal through the canonical WNT signaling pathway.

On the other hand, *CSF1R*, *TYRO3*, *SRC*, and *GSK3A* were consistently downregulated in hESCs (Fig. 2). Thus, it is proposed that these kinase genes are repressed to retain the stemness of hESCs. *CSF1R*, a tyrosine receptor kinase for *CSF1*, mediates the survival, proliferation, differentiation, and functional modulation of mature blood cells and progenitors (Barreda *et al.* 2004). *CSF1R* is expressed during early hemangioblastic differentiation (Glasker *et al.* 2006). *TYRO3* (also designated *Sky, RSE*), a member of the *AXL/TYRO3* receptor family, is abundantly expressed in differentiating cells and tissues (Crosier *et al.* 1994). *TYRO3* is essential for mammalian development and homeostatic maintenance of diverse

cell populations in nervous, reproductive, and immune systems (Lu *et al*. 1999). However, the functions of *CSF1R* and *TYRO3* in stem cells have not been established to date. The SRC family of cytoplasmic PTKs (SFKs), including *SRC*, *LYN*, *FYN*, *YES*, *LCK*, *BLK*, *HCK*, and *FGR*, plays important roles in cell proliferation, differentiation, and survival (Thomas & Brugge 1997). SFK activity is required for initiating differentiation after LIF withdrawal in murine ESCs. In particular, *SRC* is active during murine ESC differentiation (Meyn *et al*. 2005). In hESCs, *SRC* expression was increased in differentiated hESCs (Fig. 2). *GSK3A* was downregulated in undifferentiated hESCs. GSK3 contains two different isoforms, *GSK3A* and *GSK3B*, with 93% catalytic domain sequence identity, and similar biochemical and substrate properties (Ali *et al*. 2001). Inhibition of GSK3 is required to maintain pluripotency in both mouse and human ESCs (Sato *et al*. 2004). These studies are in line with our observations. Altogether, our results suggest that downregulation of these genes is a prerequisite for the stemness.

The results from comparison of gene expression profiles between different hESC lines showed wide variations (Abeyta *et al*. 2004, Skottman *et al*. 2005). These variations between the hESC lines may be the result of dissimilar culture conditions and inherent genetic variations of embryos from which ESCs were derived. To cope with this problem, we have analyzed differences and similarities in gene expression profiles of two genetically independent hESC lines cultured in identical conditions. The technique of RT-PCR with degenerate primers appears to be an effective method for identification of differentially expressed PTKs in hESCs. However, different types of PTKs may be preferentially selected by using different combinations of degenerate primers, because several options exist in the design of degenerate primers. To overcome this bias of selection of PTKs based on the type of degenerate primers, further studies will be needed to capture a large number of different PTKs.

In this study, several key PTKs regulating the stemness of hESCs were successfully identified by gene frequency analysis using degenerate PCR. This is the comprehensive report on the expression profiles of PTKs involved in the stemness of hESCs. Our findings collectively indicate that kinase gene expression is dynamic and that these PTKs are tightly regulated during the maintenance and differentiation of hESCs.

Materials and Methods

Culture and differentiation of hESCs

Two hESC lines, CHA-hES3 and SNUhES3, were employed in this study. CHA-hES3 (Ahn *et al*. 2006) and SNUhES3 (Oh *et al*. 2005) were maintained in hESC medium on a feeder layer of mitomycin C-inactivated STO cells. The hESC medium consists of DMEM/F12 (Invitrogen) supplemented with 20% serum replacement (Invitrogen), 1% non-essential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma–Aldrich), and 4 ng/ml bFGF (Invitrogen). HESCs were differentiated by treatment with 1 μM RA (Sigma–Aldrich) for 5 days. Differentiated hESCs were verified by transcriptional reduction of stem cell-specific genes, such as *POU5F1* and *NANOG*, and alkaline phosphatase (AP) staining. AP activity was measured using a specific staining kit, according to the manufacturer's protocol (Sigma–Aldrich).

Analysis of the expression profiles of PTKs in hESCs

Total RNA was extracted from hESCs using the RNeasy kit (Qiagen) and reverse transcribed (4 μg) using the Superscript First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. Degenerate PCR primers were based on the conserved DFG and DVW motifs of the tyrosine kinase catalytic domains VII and IX (Robinson *et al*. 1996, Lin *et al*. 1998). Three forward primers were designed from the following amino acid sequences: F1 (5′-K[V/I][S/C]DFG-3′), F2 (5′-K[V/I][G]DFG-3′), and F3 (5′-K[V/I][A/T]DFG-3′). One reverse primer (R) was designed from the sequence, 5′-DVW[S/A][F/Y]G-3′ (Lin *et al*. 1998). The primers used in this study are listed in Supplementary Table 1. The following PCR conditions were used: 5 cycles of 95 °C for 1 min, 42 °C for 1 min, 72 °C for 1 min, followed by 25 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products ranging from 150 to 170 bp were purified with the QIAquick Gel Extraction Kit (Qiagen) and directly subcloned into T-vector (Promega). Plasmid DNA from randomly selected positive clones were further purified with the Plasmid High-Throughput DNA Prep Kit (CoreBioSystem, Seoul, Korea) and sequenced with the ABI Prism 3700 DNA analyzer (PE Applied Biosystems, Foster City, CA, USA). Kinase gene sequences for individual clones were analyzed using BLASTN, with default parameters against the GenBank database from the National Center for Biotechnology Information. The frequency of each kinase was evaluated by dividing the number of clones of the kinase gene by the number of total clones of human origin from each sample. Significant differences in gene expression between data sets were calculated using the method of Audic & Claverie (1997) (http://www.igs.cnrs-mrs.fr/Winflat/winflat.cgi).

Quantitative real-time PCR

Gene transcripts were quantitatively detected using SYBR Green (QuantiTect SYBR Green PCR Master Mix, Qiagen), according to the manufacturer's instructions, on the 7500 Real-Time PCR System (Applied Biosystems). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/). Primer sequences are presented in Supplementary Table 1. To ensure the specificity and integrity of the PCR products, melting curve analyses were performed on all amplified products. The *GAPDH* level was used as the internal control, and fold changes were calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001). All data were obtained in triplicate.

Western blotting analysis

CHA-hES3 cells were lysed in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid) containing 1 mM PMSF and a cocktail of protease inhibitors. HESC lysates were incubated for 15 min on ice, and centrifuged at 20 000 ***g*** for 10 min at 4 °C. The supernatant was re-centrifuged for 10 min, and protein concentrations were determined using the BCA method (Pierce, Rockford, IL, USA). Total proteins (20 μg) were fractionated by SDS PAGE, and electrotransferred to polyvinylidene fluoride membranes (Millipore Corp, Bedford, MA, USA). Membranes were blocked in PBS supplemented with 0.1% Tween-20 and 5% nonfat milk for 2 h at room temperature, and incubated overnight in primary antibodies, including anti-POU5F1 (sc-8629, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MAP3K3 (1673-1, Epitomics, Burlingame, CA, USA), anti-ERBB2 (2242, Cell Signaling Technology, Beverly, MA, USA), anti-FGFR4 (sc-124, Santa Cruz Biotechnology), anti-EPHB2 (sc-1763, Santa Cruz Biotechnology), anti-CSF1R (sc-692, Santa Cruz Biotechnology), anti-TYRO3 (sc-1095, Santa Cruz Biotechnology), anti-SRC (2109, Cell Signaling Technology), and anti-GSK3A (9338, Cell Signaling Technology). After incubation with the corresponding secondary anti-rabbit HRP-conjugated or anti-mouse HRP-conjugated antibodies (Amersham), the membrane were developed using the ECL Advance kit (Amersham).

Inhibitor treatment

Inhibitors of ERBB2 (AG825) and FGFR4 (PD173074) were purchased from Calbiochem (Darmstadt, Germany) and Sigma respectively. CHA-hES3 cells were cultured in STO-conditioned medium supplemented with bFGF on Matrigel (BD Biosciences, Bedford, MA, USA) without feeder layers, as described previously (Xu *et al*. 2001). HESCs were treated with the respective inhibitors (10 μM for AG825 and 0.1 μM for PD173074) for 3 days.

Flow cytometry

CHA-hES3 cells were dissociated in Cell Dissociation Buffer (Invitrogen), filtered through a 40 μm nylon cell strainer (BD Biosciences Discovery Labware) and resuspended to about 5×10^5 cells in 100 μl diluent containing 0.1% BSA in PBS. For analyzing ESC marker expression, cells were incubated with the primary antibodies, including IgG isotype control (0.5 μg/test), AP (R&D systems, Inc., Minneapolis, MN, USA, cat. no. MAB1448, 1 μg/test), and SSEA4 (R&D systems, Inc., cat. no. MAB1435, 1 μg/test) for 30 min at 4 °C. After washing with 0.1% BSA in PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) for 30 min at 4 °C and finally stained with propidium iodide (Sigma) solution for 10 min. Cells were washed and analyzed on FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software. A total of 10 000 events were acquired, and analysis was limited to live events based on propidium iodide exclusion. The percentage of positive cells was assessed after correction for the percentage reactive to an isotype control conjugated to FITC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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