Table 2. List of DRG Genes Selectively Upregulated at Day 7 and Day 14 After Nerve Root Ligation

Gene Symbol	Gene Name	Accession No.	Functional Categorization	Day 2	Day 7	Day 14
S100a4	S100 calcium-binding protein A4	NM_012618	Cell cycle	1.7	3.2	2.6
Pycard	Apoptosis-associated speck-like protein containing a CARD	NM_172322	Cell cycle	1.7	2.4	2.1
Plk2	Polo-like kinase 2	AF136583	Cell cycle	1.4	2.1	2.7
Slc12a1	Solute carrier family 12, member 1	NM_019134	Channels/transporter	1.5	5.6	5.4
Aqp3	Aquaporin 3	L35108	Channels/transporter	1.3	2.7	2.9
Ctss	Cathepsin S	NM_017320	Enzyme/metabolism	1.7	4.9	3.5
Dpp6	Dipeptidylpeptidase 6	M76426	Enzyme/metabolism	1.1	4.5	4.4
Adn	Adipsin	S73894	Enzyme/metabolism	1.6	3.9	4.4
Mmp2	Matrix metallopeptidase 2	U65656	Enzyme/metabolism	1.2	3.0	2.6
Ctsk	Cathepsin K	AF010306	Enzyme/metabolism	1.4	2.7	2.4
Cnp1	Cyclic nucleotide phosphodiesterase 1	L16532	Enzyme/metabolism	1.1	2.6	2.4
Slpi	Secretory leukocyte peptidase inhibitor	AF151982	Enzyme/metabolism	1.6	2.6	2.3
Plat	Plasminogen activator, tissue	NM_013151	Enzyme/metabolism	1.5	2.5	2.6
Pld1	Phospholipase D1	U69550	Enzyme/metabolism	1.4	2.4	2.2
Cp	Ceruloplasmin	NM_012532	Enzyme/metabolism	1.2	2.4	2.2
Hspa2	Heat shock protein 2	NM_021863	Enzyme/metabolism	1.2	2.4	2.1
Xdh	Xanthine dehydrogenase	NM_017154	Enzyme/metabolism	1.4	2.4	2.4
Dnah7	Dynein, axonemal, heavy polypeptide 7	D26498	Enzyme/metabolism	1.6	2.2	2.1
Mmp10	Matrix metallopeptidase 10	M65253	Enzyme/metabolism	1.1	2.2	2.4
Ugdh	UDP-glucose dehydrogenase	AB013732	Enzyme/metabolism	1.7	2.1	2.3
Fap	Fibroblast activation protein	NM_138850	Enzyme/metabolism	1.4	2.0	2.0
Entpd2	Ectonucleoside triphosphate diphosphohydrolase 2	AF276940	Enzyme/metabolism	1.0	2.0	2.2
Mdk	Midkine	AB025023	Growth factors/cytokines	1.3	2.3	2.1
Igf1	Insulin-like growth factor I	M81184	Growth factors/cytokines	1.5	2.3	2.6
Tgfbi	Transforming growth factor, beta induced	AF305713	Growth factors/cytokines	1.5	2.2	2.0
C1qb	Complement component 1, q subcomponent, beta polypeptide	NM_019262	Immune	1.9	3.5	2.4
Cxcl9	Chemokine (C-X-C motif) ligand 9	NM_145672	Immune	1.0	3.5	3.5
Cfh	Complement component factor H	NM_130409	Immune	1.5	2.7	2.8
Cd86	Cd86 antigen	D50558	Immune	1.8	2.1	2.4
Apod	Apolipoprotein D	NM_012777	Signal transduction	1.7	4.7	3.3
Lgp85	85 kDa sialoglycoprotein	D10587	Signal transduction	1.7	3.1	2.6
Gng11	Guanine nucleotide binding protein (G protein), γ11	AF257110	Signal transduction	1.6	3.1	3.1
Gfra1	Glial cell line derived neurotrophic factor family receptor α	NM_012959	Signal transduction	1.9	3.1	2.3
Cd53	CD53 antigen	NM_012523	Signal transduction	1.7	2.8	2.3
Ramp1	Receptor (calcitonin) activity modifying protein 1	AB042887	Signal transduction	1.4	2.7	2.5
Csrp2	Cysteine and glycine-rich protein 2	U44948	Signal transduction	1.2	2.6	2.6
Ogfr	Opioid growth factor receptor	AF156878	Signal transduction	1.4	2.4	2.3
Vcam1	Vascular cell adhesion molecule 1	NM_012889	Signal transduction	1.7	2.2	2.1
Sostdc1	Uterine sensitization-associated gene 1 protein	NM_153737	Signal transduction	0.8	2.2	2.4
Slfn3	Schlafen 3	AF168795	Signal transduction	1.7	2.1	2.1
Col3a1	Collagen, type III, α1	M21354	Structure	1.8	4.0	2.9
Serpinh1	Serine (or cysteine) proteinase inhibitor, clade H, member 1	NM_017173	Structure	1.9	2.6	2.4
Lum	Lumican	X84039	Structure	1.6	2.4	2.3
Col5a1	Collagen, type V, α1	AF272662	Structure	1.5	2.3	2.5
Vil2	Villin 2	X67788	Structure	1.8	2.3	2.1
Col1a1	Collagen, type 1, α1	Z78279	Structure	1.2	2.2	2.0
DLP2	Dynein-like protein 2	D26493	Structure	1.5	2.1	2.2
Apobec1	Apolipoprotein B editing complex 1	NM_012907	Transcription/translation	1.6	3.3	3.0
Mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B	U56241	Transcription/translation	1.4	2.4	2.4
Nupr1	Nuclear protein 1	AF014503	Transcription/translation	1.1	2.3	2.0
H3f3b	H3 histone, family 3B	X73683	Transcription/translation	1.6	2.2	2.0
Irf1	Interferon regulatory factor 1	NM_012591	Transcription/translation	1.7	2.1	2.0
Meox2	Mesenchyme homeo box 2	NM_017149	Transcription/translation	1.5	2.1	2.4
Btg1	B-cell translocation gene 1, anti-proliferative	L26268	Transcription/translation	1.7	2.1	2.9
Lhx2	LIM homeobox protein 2	L06804	Transcription/translation	1.6	2.0	2.4
Drcf5	Developmentally-regulated cardiac factor	U95001	Unknown	1.6	2.5	2.7

The average Cy5/Cyt 3 ratio of gene in triplicate microarray experiments were calculated. Genes showing the ratio of two or more at day 7 and day 14, but not day 2 were listed.

cluster composed of the day-2 DRG samples. Subsequently, we identified 89 genes, including 16 genes that were upregulated only at day 2 after nerve root ligation, 56 genes that were upregulated at days 7 and 14, and another 17 genes that showed consistent upregulation from day 2 to day 21. These findings indicate the involvement of distinct sets of genes in the early phase (day 2) and the later phase (day 7 and later) of development of lumbar radiculopathy. The number of upregulated genes was larger in the midphase than in the early phase, cor-

Table 3. List of DRG Genes Continuously Upregulated From Day 2 to Day 21 After Nerve Root Ligation

Gene symbol	Gene Name	Accession No.	Functional Categorization	Day 2	Day 7	Day 14	Day 21
Kif11	Kinesin family member 11	AF035955	Cell cycle	2.5	3.2	3.0	2.1
Lyz	Lysozyme	NM_012771	Enzyme/metabolism	2.1	6.2	5.0	3.6
Crabp2	Cellular retinoic acid binding protein 2	U23407	Enzyme/metabolism	3.1	3.3	3.0	2.1
Enpp3	Ectonucleotide pyrophosphatase/ phosphodiesterase 3	NM_019370	Enzyme/metabolism	2.7	3.8	4.8	2.8
Reg3g	Regenerating islet-derived 3γ	L20869	Enzyme/metabolism	2.7	2.2	4.9	2.7
A2m	α-2-macroglobulin	NM_012488	Enzyme/metabolism	5.1	11.6	10.8	2.3
Ifitm2	Interferon induced transmembrane protein 2 (1–8D)	AF164040	Immune	2.0	3.1	2.1	2.1
Gbp2	Guanylate nucleotide binding protein 2	M80367	Immune	3.1	3.9	4.1	2.1
Ifitm3	Interferon induced transmembrane protein 3	AF164039	Immune	2.3	3.3	2.6	2.3
Fcgr2b	Fc receptor, IgG, low affinity IIb	X73371	Immune	4.4	3.1	2.8	2.4
Gpnmb	Glycoprotein (transmembrane) nmb	AF184983	Signal transduction	2.3	3.6	4.8	2.9
Cdh4	Cadherin 4	D86742	Signal transduction	2.0	3.4	2.6	2.0
Vim	Vimentin	X62952	Structure	2.7	4.8	2.8	2.2
Thbs4	Thrombospondin 4	X89963	Structure	2.1	7.2	5.8	2.3
Cthrc1	Collagen triple helix repeat containing 1	NM_172333	Structure	4.7	8.7	10.4	2.5
Fn1	Fibronectin 1	NM_019143	Structure	2.8	3.3	3.1	2.1
Col18a1	Procollagen, type XVIII, α1	AF189709	Structure	3.5	6.8	5.4	2.4

The average Cy5/Cyt 3 ratio of gene in triplicate microarray experiments were calculated. Genes showing the ratio of 2 or more continuously from day 2 to day 21 were listed.

responding to the painful behavior of the nerve-ligated rats which peaked during the midphase.[15]

Functional categorization revealed dominantly upregulated gene categories in each group. They included transcription/translation, signal transduction, and enzyme/metabolism in the early phase and enzyme/metabolism, signal transduction, transcription/translation, and structure in the midphase. Consistent with our findings, genes categorized into enzyme/metabolism, signal transduction, transcription/translation, and structure were also dominantly upregulated in the dorsal horn of the spinal cord 7 and 14 days after nerve root ligation in a rat model.[4]

Based on clustering analysis, we classified genes into the early phase, midphase, and continuous group. In this regard, genes upregulated on day 14 or later may be classified as the late phase. We found only 2 genes in this group, including EGF-like domain, multiple 3 and coxsackie virus and adenovirus receptor.

Genes upregulated in the DRG with nerve root ligation may contribute to a variety of biologic processes including pain signaling, inflammation, and regeneration of the injured nerve. Of these, the genes involved in pain signaling and inflammation may serve as potential targets of pharmacological or genetic approach for lumbar radiculopathy. This idea is well exemplified by prostaglandin-endoperoxide synthase 2, which is upregulated in the early phase of our model. Prostaglandin-endoperoxide synthase 2 is known as cyclooxygenase 2 (COX-2). Involvement of COX-2 in acute pain and inflammation has been established.[18,19] Also, therapeutic efficacy of a selective COX-2 inhibitor has been demonstrated in rat radiculopathy models.[20,21] Besides prostag-

Table 4. Percentage of Genes Classified by Functional Category

	Early	Mid	Consistent
Cell cycle	6	5	6
Channels/transporters	6	4	0
Enzyme/metabolism	13	31	29
Growth factors/cytokines	6	5	0
Immune	6	7	24
Signal transduction	19	20	12
Structure	0	13	29
Synaptic	6	0	0
Transcription/translation	31	15	0
Unknown	6	2	0

Table 5. Real-Time Quantitative PCR Analysis of Day 7 DRG

Gene Name	Probe No.*	Ligation	Sham
Apolipoprotein D	Rn00562832_m1	12.0**	1.0
CD53 antigen	Rn00560957_m1	4.7	0.9
Aquaporin 3	Rn00581754_m1	64.0	0.8
Receptor (calcitonin) activity modifying protein 1	Rn00581278_m1	2.7	0.5
Secretory leukocyte peptidase inhibitor	Rn00670378_m1	15.9	0.7
Plasminogen activator, tissue	Rn00565767_m1	3.3	0.9
Heat shock protein 2	Rn00434069_s1	3.0	1.2
Xanthine dehydrogenase	Rn00567654_m1	3.9	0.9
V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B	Rn00709456_s1	2.7	1.3
Opioid growth factor receptor	Rn00584280_m1	1.6	1.0
Collagen, type V, α1	Rn00593170_m1	2.6	1.0
Uterine sensitization-associated gene 1 protein	Rn00596672_m1	2.7	0.9
Polo-like kinase 2	Rn00582709_m1	3.2	1.1
cd86 antigen	Rn00571654_m1	6.4	0.9
UDP-glucose dehydrogenase	Rn00580047_m1	4.4	1.1
B-cell translocation gene 1, anti-proliferative	Rn00820872_g1	3.8	0.8
Ectonucleoside triphosphate diphosphohydrolase 2	Rn00596961_m1	3.3	1.0

*The catalog no. of TaqMan probes (Applied Biosystems).
**The mean relative expression values of three root-ligated rats and sham-operated rats were described, respectively.

landin-endoperoxide synthase 2, calcium-activated chloride channel may contribute to pain signaling in lumbar radiculopathy, because calcium-activated chloride current[22] has been shown to increase after axotomy in rats. In contrast to these molecules, heme oxygenase (decycling) 1 serves cytoprotective actions in several pathologic conditions including inflammation[23] and sciatic nerve injury.[24] Thus, there may be therapeutic benefits with heme oxygenase (decycling) 1 in lumbar radiculopathy. Among 56 genes that were upregulated in the midphase, the involvement of cathepsin S in neuropathic pain has been recently reported.[25,26] Given the fact that inhibition of cathepsin S led to reversal of neuropathic pain after peripheral nerve injury, this approach can also be applied to neuropathic condition subsequent to lumbar radiculopathy.

The present study has several limitations, including–(i) imperfect validity of microarray results (94%), (ii) placement of sham-operated controls only at 1 time point (day 7), and (iii) lack of immunohistochemical or *in situ* hybridization studies that reveals cell types expressing genes upregulated. Nevertheless, this is the first comprehensive analysis of gene expression in the DRG adjacent to nerve root ligation. The present study serves as the initial step toward identification of therapeutic targets for lumbar radiculopathy and development of molecularly targeted therapy.

■ Key Points

- DNA array analysis was conducted for the DRG adjacent to the injured nerve root in a rat lumbar radiculopathy model.
- Of 7793 genes analyzed, 16 genes were upregulated in the DRG on day 2 after nerve root ligation (early phase group), 56 genes on both days 7 and 14 (midphase group), and 17 genes from day 2 to day 21 (continuous group).
- Dominantly upregulated gene categories were transcription/translation in the early phase group, enzyme/metabolism in the midphase group, and structure in the continuous group.

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p63 Induces CD4$^+$ T-Cell Chemoattractant TARC/CCL17 in Human Epithelial Cells

Terufumi Kubo, Shingo Ichimiya, Akiko Tonooka, Tsutomu Nagashima, Tomoki Kikuchi, and Noriyuki Sato

To preserve immunosurveillance, epithelial cells support T-cell trafficking toward inflammatory foci. However, how epithelial cells are enrolled in recruiting T cells has not been fully elucidated. In this study we investigated the function of p63, a p53 family member, in the regulation of the expression of various types of chemokine ligands by focusing on the property of p63 as an epitheliotropic transcription factor. As assessed by experiments using three different human epithelial cell lines with small-interfering RNAs or plasmids of p63, certain CC chemokine ligands were found to be under the control of p63. In these CC chemokine ligands, p63 had the common capacity to upregulate TARC/CCL17 in the different cell lines, whose receptor CCR4 was preferentially presented on CD4$^+$ T cells such as memory, regulatory, IL-17-producing and type II helper T cells. More interestingly, when cells were stimulated with transforming growth factor-β (TGF-β) or epidermal growth factor (EGF) as observed during tissue repair process, the expression of p63 and TARC/CCL17 was concomitantly suppressed. This implies that, in local inflammatory regions with general epithelial tissue remodeling, the p63-TARC/CCL17 axis may participate in the engagement of efficient immune reactions by specified T-cell subsets.

Introduction

It is well recognized that epithelial cells play an important role in the immune system as the front line of defense against external pathogens (Hayday and others 2001). Anatomically, epithelial cells of tissues such as lung, skin, and intestines tightly connect to each other to form a sheet structure with various junctional molecules (Tsukita and Furuse 2000). These epithelial networks eventually help defend against pathogens and also evoke cellular and molecular interactions of immune responses by recruiting a multitude of inflammatory cells like lymphocytes and dendritic cells (von Andrian and Mackay 2000). In addition to the mechanisms of innate immunity, acquired or adaptive immunity is essential for specific responses to various immunologic insults around epithelial tissues. The mechanism of homing of inflammatory cells to epithelial cells has been extensively investigated and soluble factors, including chemokine ligands, are known to have a pivotal function, but the secretion mechanism by which epithelial cells foster regional lymphocytes is still unclear.

Chemokine ligands are secreted polypeptides that trigger an elaborate process whereby inflammatory cells presenting chemokine receptors of seven-transmembrane G-protein-coupled receptors can promptly channel them to the foci requiring immune and inflammatory reactions (Kunkel and Butcher 2002; Milligan and Smith 2007). Based on their structural characteristics with regard to the positions of a cysteine residue, chemokine ligands are classified into two major subfamilies: CC and CXC chemokine ligands. To date many different CC and CXC chemokine ligands have been discovered and some of them derived from epithelial tissues are known to play cardinal roles in the emergence of allergic or atopic inflammatory disorders and cancer development (Juremalm and others 2005; Ruffini and others 2007).

Accumulating evidence has indicated that p63, a p53-like molecule, is a usual epithelial constituent and determines properties of epithelial stem cells (Truong and others 2006). Indeed, loss of p63 leads to severe epithelial tissue hypoplasia due to the inhibition of both stratification and differentiation of keratinocytes (Koster and others 2007). Various genes, encoding JAG1, PERP, p21, or 14–3-3σ, are transactivated by p63, but it is not well investigated whether p63 is involved in the expression of chemokine ligands in epithelial cells (Sasaki and others 2002; Westfall and others 2003; Ihri and others 2005).

In this study we assessed the role of p63 in the regulation of the transcription of a series of chemokine ligands on three different types of human epithelial cells with

Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan.

small-interfering RNAs (siRNAs) or plasmid DNAs of p63. We clearly found some CC chemokine ligands that could be regulated by p63. Among the chemokine ligands we investigated, the only one that p63 could commonly upregulate in the three epithelial cell lines we investigated was thymus and activation-regulated chemokine (TARC)/CCL17, a strong chemoattractant of $CD4^+$ T cells like memory, regulatory and type II Th as well as Th17 cells (Vestergaard and others 2000; Baekkevold and others 2005; Hirahara and others 2006; Lim and others 2008). Tissue remodeling–associated cytokines such as transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) suppressed the expression of p63 of epithelial cells as under the repair process (Kurokawa and others 2006). It was of great interest that, in accordance with the downregulation of p63, cells exposed to these cytokines showed the suppression of TARC/CCL17 as expected. This may illustrate an instructive mechanism mediated by p63, by which epithelial tissues could retain $CD4^+$ T-cell populations responsible for immunomodulation ready to be mobilized at the sites of immune reactions during the physiological healing process of epithelial tissues. As TARC/CCL17 is well known to be involved in the emergence of allergic bronchitis and allergic or atopic dermatitis, our observations may lead to further understanding of the mechanisms of such immune-related disorders (Sekiya and others 2000; Campbell and others 2007; Furusyo and others 2007).

Materials and Methods

Cell lines and cell culture

Human LC817, HaCaT, and 293 epithelial cells were maintained in modified Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated bovine calf serum, 50 μg/mL streptomycin, and 100 U/mL penicillin. All cells were cultured at 37°C in a humidified atmosphere in 5% CO_2.

Antibodies and immunohistochemistry

The antibody used was a rabbit anti-p63 polyclonal antibody (H-137; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The procedures for immunofluorescence have been previously described (Kikuchi and others 2004). Signals were detected under an immunofluorescence microscope (IX71, Olympus).

RT-PCR and real-time RT-PCR

Primer pairs were selected using Primer3 software on messenger RNA sequences based on the National Center for Biotechnology Information database. Sequences of the primer pairs were finally determined after reference to the original genomic organization presented in the Ensembl database (Sanger Centre), as summarized in Table 1. Real-time polymerase chain reaction (PCR) was performed as described in the manufacturer's protocol for Assays-on-Demand Gene Expression products (Applied Biosystems). To compare the levels of expression of genes, the ΔΔCT method was employed to analyze triplicate specimens (Livak and Schmittgen 2001).

Transfection of siRNAs and plasmid DNAs

Cells were cultured at a density of 2×10^5 cells/well in a 6-well plate in 2.5-mL culture medium. After 24 h, the culture medium was replaced by medium containing a complex of the siRNAs specific for p63 (B-Bridge) and Lipofectamine RNAi MAX (Invitrogen), giving a final concentration of siRNA of 20 nM following the manufacturer's instructions. After 48 h, cells were harvested to be subjected to real-time PCR analysis. As with transfection of siRNAs, cells were prepared prior to transfection. After 24 h, 250 ng of pcDNA3.1 harboring cDNA encoding p63 (pcDNA3.1-p63) was transiently transfected using Lipofectamine 2000 reagent (Invitrogen). After 48 h, cells were harvested and analyzed.

Cell stimulation

Cells were cultured at a density of 2×10^5 cells 24 h before stimulation. Then the culture medium was replaced by a medium containing 10 U/mL TGF-β (Sigma-Aldrich) or 10 U/mL EGF (Sigma-Aldrich). After 48 h total RNA was harvested from cells to be employed in subsequent real-time PCR analysis.

Results

Chemokine ligands regulated by p63 in LC817 pulmonary epithelial cells

To address the question of whether p63 was involved in the regulation of chemokines of epithelial cells, we initially established a specialized cellular state with suppressed levels of p63 by employing the siRNA technique. When the designated siRNA from a sequence encoding p63 was transiently introduced into LC817 pulmonary epithelial cells that constitutively expressed p63, it was downregulated at transcription and protein levels as well (Fig. 1A). Using these LC817 cells, reverse transcriptase (RT)-PCR analyses of CC chemokine ligands, including CCL1 to CCL28, indicated that the expression of TARC/CCL17, CCL22, CCL25, and CCL28 was likely to be upregulated by p63 (Fig. 1B). CCL5 was also under the control of p63, but was negatively regulated. A series of CXC chemokine ligands was also investigated in LC817 cells, though there were no significant changes of any type of CXC chemokine ligand, including CXC1 to CXC16 at the transcription level (Fig. 1C). Thus p63 could regulate the levels of CC chemokine ligands of CCL5, TARC/CCL17, CCL22, CCL25, and CCL28 but not CXC chemokine ligands in LC817 cells.

Chemokine ligands regulated by p63 in HaCaT epidermal cells

To evaluate the results obtained from the analysis of LC817 cells, we further examined HaCaT epidermal cells, which also endogenously expressed p63. Prior to RT-PCR analyses, it was also confirmed that HaCaT cells with the siRNAs of p63

TABLE 1. PRIMER SEQUENCES USED IN THIS STUDY.

		(Forward primer)			(Reverse primer)		
Gene name	*Length (bp)*	*sequence (5'–3')*	*Temp(°C)*	*Position*	*sequence (5'–3')*	*Temp(°C)*	*Position*
CCL1	163	catttgcggagcaagagatt	60.4	exon2	tgcctcagcatttttctgtg	60.0	exon3
CCL2	171	ccccagtcacctgctgttat	60.0	exon2	tggaatcctgaacccacttc	59.9	exon3
CCL3	198	tgcaaccagttctctgcatc	60.0	exon1	tttctggacccactcctcac	60.1	exon3
CCL4	211	aagctctgcgtgactgtcct	60.2	exon1	gcttgcttcttttggtttgg	59.9	exon3
CCL5	150	cgctgtcatcctcattgcta	60.0	exon1	gagcacttgccactggtgta	59.9	exon2
CCL7	179	atgaaagcctctgcagcact	60.2	exon1	ggacagtggctactggtggt	60.0	exon2
CCL8	192	aatgtcccaaggaagctgtg	60.1	exon2	gggaggttggggaaaataaa	60.0	exon3
CCL11	233	agaaaccaccacctctcacg	60.2	exon1	cacagctttctggggacatt	60.1	exon2
CCL13	245	atctccttgcagaggctgaa	60.1	exon2	agaagaggaggccagaggag	60.1	exon3
CCL14/15	214	tcccgtgttcactcatgaaa	60.1	exon3	tcagaggctcaggaggtgtt	60.0	exon4–5
CCL16	242	ctgccctgtctctccttgtc	60.0	exon1	ctcttggacccagtcgtcat	60.1	exon3
CCL17	163	cttctctgcagcacatccac	59.6	exon1	ctgccctgcacagttacaaa	59.9	exon3
CCL18	150	agctctgctgcctcgtctat	59.4	exon2	cccacttcttattggggtca	59.8	exon3
CCL19	172	atccctgggtacatcgtgag	59.8	exon2	gcttcatcttggctgaggtc	60.0	exon3
CCL20	198	tttattgtgggcttcacacg	59.6	exon2	tgggctatgtccaattccat	60.2	exon3
CCL21	220	caagcttaggctgctccatc	60.1	exon2	tcagtcctcttgcagcctttt	60.1	exon3
CCL22	212	cgcgtggtgaaacacttcta	59.9	exon2	ataatggcagggaggtaggg	60.2	exon3
CCL23	173	tttgaaacgaacagcgagtg	60.0	exon3	tgtgtcccttcaccttgaca	60.1	exon4
CCL24	228	gccttctgttccttggtgtc	59.7	exon1	tgtacctctggacccactcc	60.0	exon3
CCL25	198	acaggaaggtgtgtgggaac	59.9	exon3	tactgctgctgatgggattg	59.8	exon4
CCL26	163	ggaggagtttgggagaaacc	59.9	exon2	tgtggctgtattggaagcag	59.9	exon3
CCL27	191	agcactgcctgctgtactca	59.8	exon2	tcttggtgctcaaaccactg	59.9	exon3
CCL28	188	gctgatggggattgtgactt	59.9	exon2	ccctgatgtgccctgttact	60.0	exon3
CXCL1	171	agggaattcaccccaagaac	60.2	exon2	tggatttgtcactgttcagca	60.3	exon2–3
CXCL2	172	gcagggaattcacctcaaga	60.2	exon2	ggatttgccatttttcagc	61.0	exon3–4
CXCL3	172	gcagggaattcacctcaaga	60.2	exon2	ggtgctccccttgttcagt	61.0	exon3–4
CXCL4	174	gctgctcctgccacttgt	60.2	exon1	ttcagcgtggctatcagttg	60.0	exon2–3
CXCL5	171	gcaaggagttcatcccaaaa	60.1	exon2	ttgtttccaccgtccaaaat	60.2	exon3–4
CXCL6	158	gtcctgtctctgctgtgctg	59.8	exon2	aacttgcttcccgttcttca	59.9	exon3
CXCL7	207	tcctccaccaaaggacaaac	59.9	exon1	tttcctcccatccttcagtg	60.0	exon3
CXCL8	196	gtgcagttttgccaaggagt	60.3	exon1–2	ctctgcacccagttttcctt	59.3	exon4
CXCL9	166	ttttcctcttgggcatcatc	60.0	exon1	tcaattttctcgcaggaagg	60.3	exon2
CXCL10	172	ctgtacgctgtacctgcatca	59.9	exon2	ttcttgatggccttcgattc	60.2	exon3
CXCL11	175	agaggacgctgtctttgcat	60.0	exon2	taagccttgcttgcttcgat	60.1	exon3
CXCL12	161	tcagcctgagctacagatgc	59.3	exon2	ctttagcttcgggtcaatgc	59.9	exon3
CXCL13	151	gcttgaggtgtagatgtgtcca	60.2	exon2	tgagggtccacacacacaat	59.9	exon3
CXCL14	152	aagctggaaatgaagccaaa	59.8	exon2	ttccaggcgttgtaccactt	60.6	exon3
CXCL16	160	tctccggaaacacctgagag	60.4	exon2	cacaatccccgagtaagcat	60.0	exon4
p63	163	gaaacgtacaggcaacagca	59.9	exon10	gctgctgagggttgataagc	60.0	exon11

expressed p63 to a lesser extent than control cells (Fig. 2A). As assessed by RT-PCR analyses, CCL7, TARC/CCL17, and CCL26 were upregulated by p63 in HaCaT cells (Fig. 2B). In HaCaT cells, CCL5 was also slightly upregulated and CCL25 was downregulated by p63, though p63 insignificantly affected the expression of CCL22 and CCL28. These results, except for TARC/CCL17, did not match those of LC817 cells, suggesting that the manner of p63 regulation of CC chemokine ligands probably varied by epithelial cell type. Thus TARC/CCL17 was thought to be a particular CC chemokine ligand with a mechanism of transcription regulated by p63 that might be common in usual epithelial cells.

Excess amounts of p63 induce TARC/CCL17 in 293 epithelial cells

Following these results, we next employed an alternative approach to determine whether CC chemokine ligands CCL5, CCL7, TARC/CCL17, CCL22, CCL25, CCL26, and CCL28 were regulated by p63. As 293 epithelial cells did not endogenously express p63, the cells were transiently transfected with p63 plasmids. Interestingly RT-PCR analyses of 293 cells with excess p63 demonstrated high amounts of TARC/CCL17 (Fig. 3). The levels of CCL5 and CCL26 were relatively decreased in 293 cells with excess p63. This was

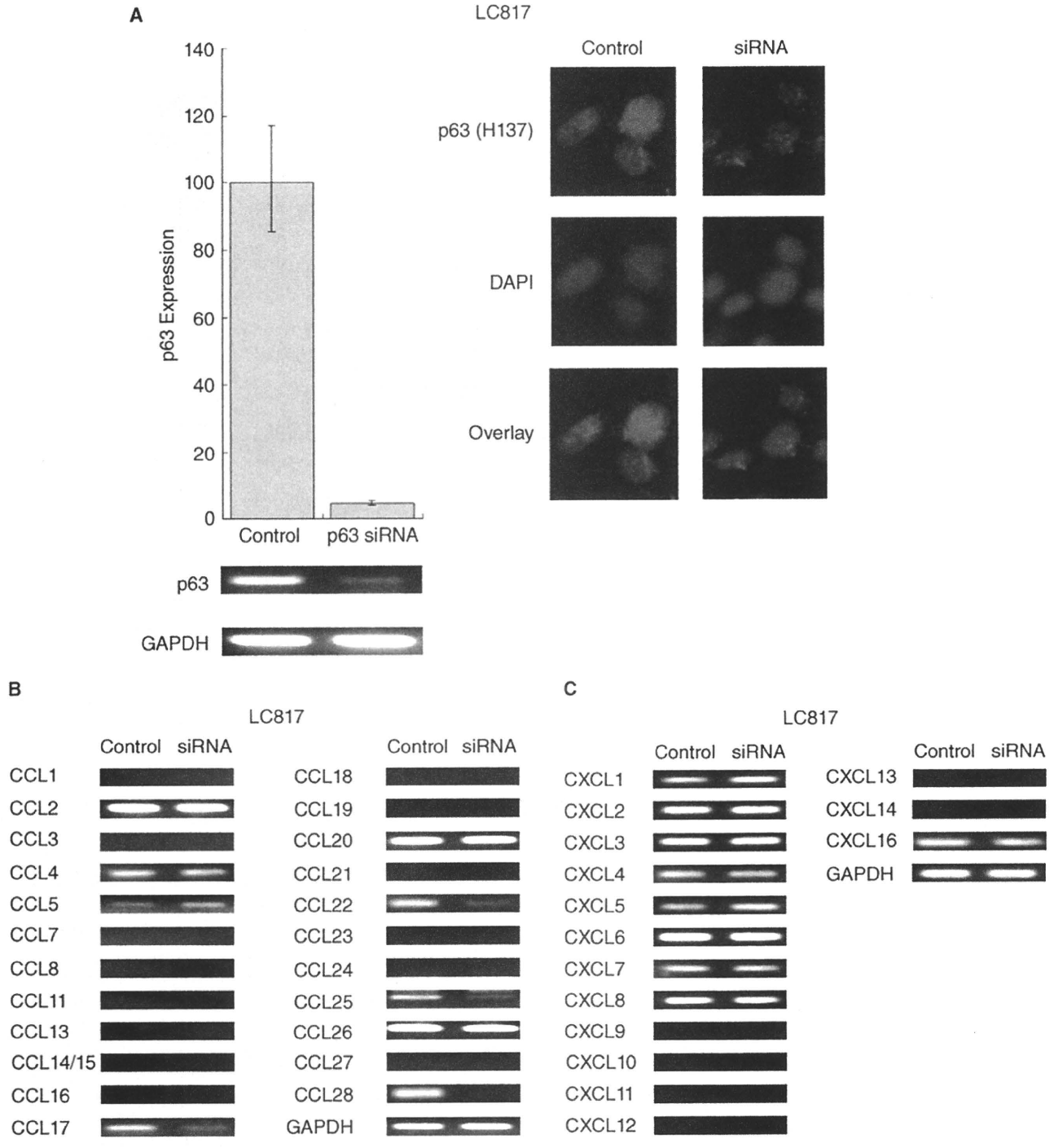

FIG. 1. Transcription analysis of LC817 pulmonary epithelial cells. (**A**) Transfection of siRNAs specific for p63 resulted in the downregulation of p63 in LC817 cells. Left panels show real-time PCR analysis of cDNAs derived from cells with siRNA specific for GFP or siRNA specific for p63. Simultaneously, cDNAs were checked by RT-PCR analysis of p63. Right panels show immunofluorescence analysis of cells with siRNA of GFP or p63 (depicted as control and siRNA, respectively), where p63 is visualized by H137 pAb in red and the nucleus by DAPI in blue. (**B** and **C**) Transcription profiles as assessed by RT-PCR analysis of CC and CXC chemokine ligands, respectively. Control, cells transfected with GFP siRNAs; p63 siRNA or siRNA, cells with p63 siRNAs; GAPDH, a template control.

similarly found in LC817 and HaCaT cells, suggesting that p63 was probably associated with the control of the expression of CCL5 through various factors depending on the cells. In 293 cells, the levels of CCL22, CCL25, and CCL28 were not altered and CCL7 seemed not to be associated with p63. Collectively, the mechanism by which p63 positively

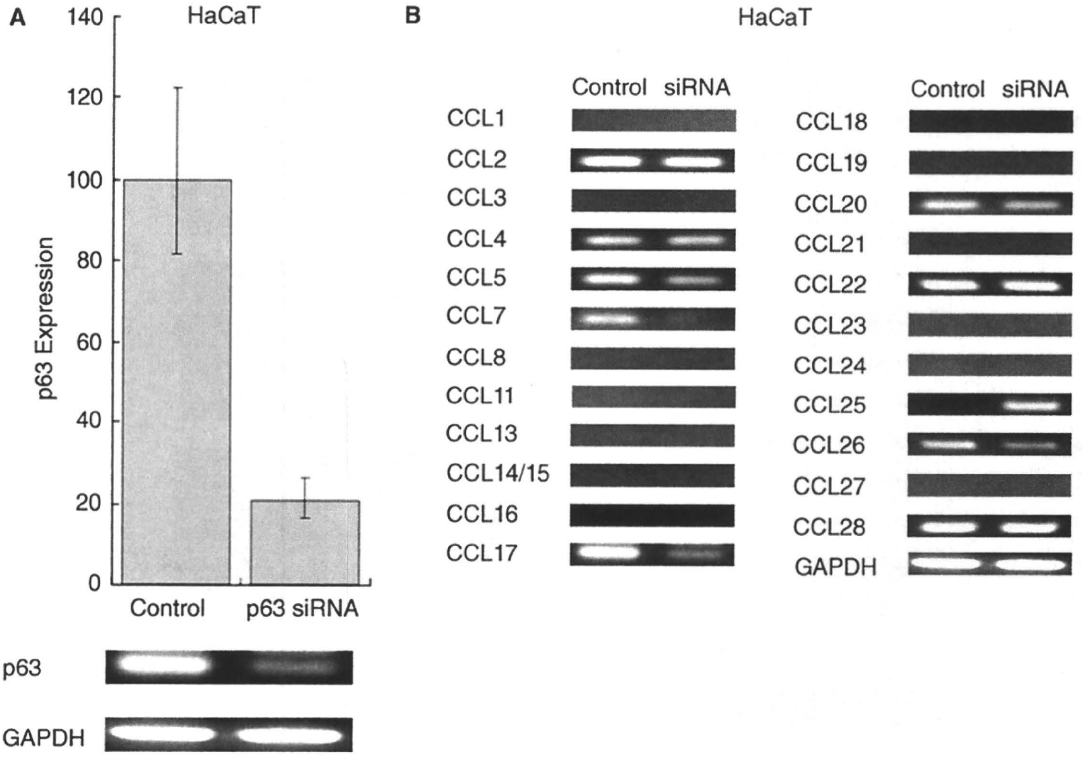

FIG. 2. Transcription analysis of HaCaT epidermal cells. (**A**) Transfection of siRNAs specific for p63 resulted in the downregulation of p63 in HaCaT cells. Real-time PCR analysis of cDNAs derived from cells with siRNA specific for GFP and with siRNA specific for p63. Simultaneously, cDNAs were evaluated by RT-PCR analysis of p63 and GAPDH as a template control. (**B**) Transcription profiles as assessed by RT-PCR analysis of CC chemikine ligands in HaCaT cells. Control, cells transfected with GFP siRNAs; siRNA, cells transfected with p63 siRNAs; GAPDH, a template control.

induced the expression of TARC/CCL17 would generally appear to be shared by human epithelial cells. It was also observed that CCL5 and CCL26 were under the control of p63 in specific types of epithelial cells.

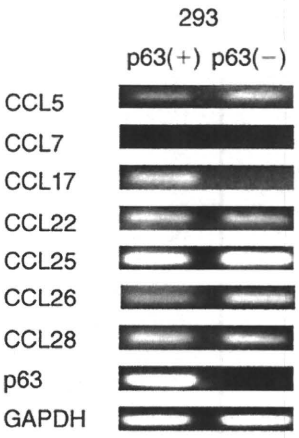

FIG. 3. Transcription analysis of CCL5, CCL7, CCL17, CCL22, CCL25, CCL26, and CCL28 in 293 epithelial cells with transiently introduced mock pcDNA3.1 or p63-pcDNA3.1 (depicted as p63(−) or p63(+), respectively) by RT-PCR. GAPDH, a template control.

TGF-β and EGF downregulate p63 and TARC/CCL17

It is well recognized that inflammatory responses are usually accompanied by inflammatory healing reactions around local epithelial tissues. A complex mechanism with multiple inflammatory cells underlays these reactions, in which TGF-β and EGF have pivotal roles in such remodeling during immune responses. When epithelial remodeling around inflammatory foci occurs, the levels of p63 are mostly suppressed in epithelial cells (Bamberger and others 2005; Kurokawa and others 2006). To examine the physiological role of p63 in the context of TARC/CCL17 expression, HaCaT cells were stimulated with TGF-β or EGF. As under inflammatory conditions, the levels of p63 were inhibited in these cells down to ~40–50% of the control levels (Fig. 4). As expected due to the downregulation of p63, the levels of TARC/CCL17 were significantly decreased to 5–10% of the control levels. This may imply physiological association of TGF-β and EGF with the regulation of TARC/CCL17 through p63.

Discussion

In this study, we first identified a role of the p63 molecule in the positive regulation of TARC/CCL17 in human

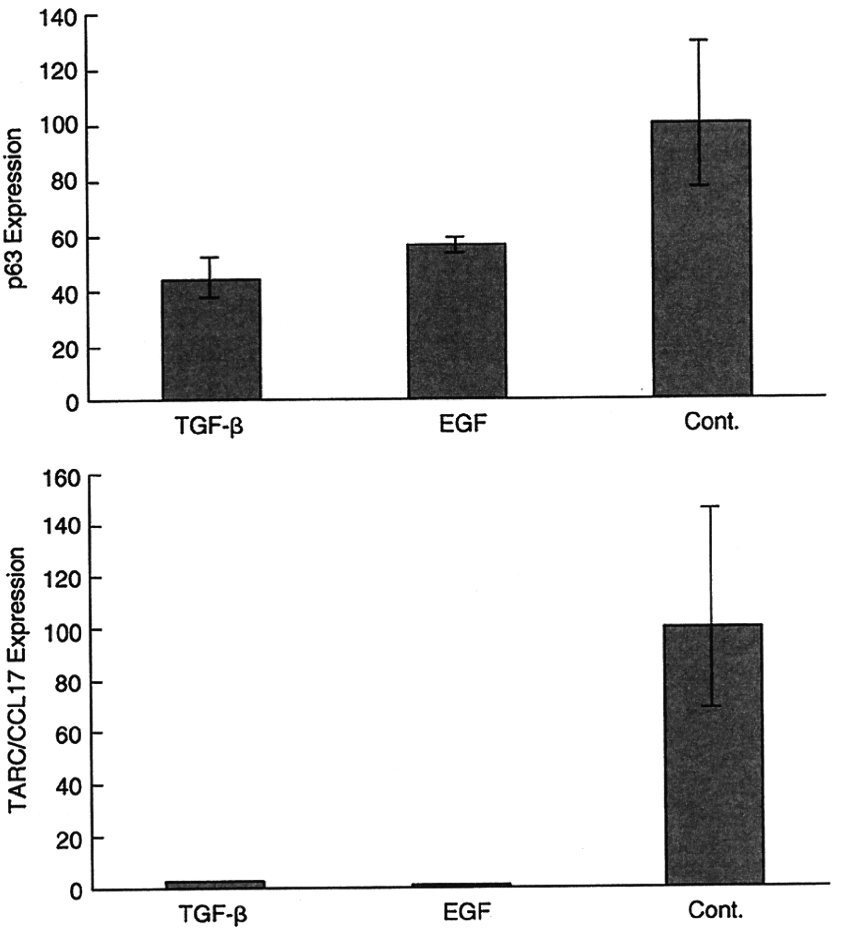

FIG. 4. Transcription analysis of p63 and TARC/CCL17 in HaCaT cells stimulated with TGF-β or EGF associated with tissue remodeling by real-time PCR. *Upper and lower panels* show levels of transcription of p63 and TARC/CCL17, respectively.

epithelial cells. The transcripts of TARC/CCL17 are exclusively detected in epithelial tissues, including lung and small and large intestines as well as thymus, suggesting that TARC/CCL17 mainly acts as a chemokine of epithelial origin (Imai and others 1996). Because p63 shows an expression profile with TARC/CCL17 in various human epithelial tissues, our results are compatible with the functional correlations implied by the expression profiles of p63 and TARC/CCL17 (Schmale and Bamberger 1997; Kikuchi and others 2004). Similar to the adhesion molecules, chemokines and their surface receptors can be up- or downregulated as cells undergo differentiation or stimulation, allowing lymphocytes to coordinate their own pathway with their immunologic functions. In this context, it is intriguing that TGF-β and EGF suppressed the levels of TARC/CCL17, probably through downregulation of p63, as during the inflammatory healing process, where external pathogens can enter into the tissues from the openings of broken epithelial cell sheets. This might imply that the p63-TARC/CCL17 axis participates in the homing and release of particular T-cell subsets and then engages efficient immune reactions at local inflammatory regions with epithelial tissue remodeling.

Since $CD4^+$ T cells chiefly conduct immunosurveillance, they have to be mobilized to the sites where antigens are localized and presented through a major histocompatibility complex or CD1 (Bendelac and others 2007). TARC/CCL17 specifically binds to the CC chemokine receptor CCR4 on the surface of the $CD4^+$ T cell. It is noteworthy that memory and regulatory T cells, Th17 and Th2 $CD4^+$ T cells, possess CCR4 and are possibly able to home through TARC/CCL17 in skin, lung, and tumor tissues (Vestargaard and others 2000; Backkevold and others 2005; Hirahara and others 2006; Lim and others 2008). Active involvement of TARC/CCL17 has also been reported in the emergence of allergic as well as atopic disorders in skin and lung (Sekiya and others 2000; Campbell and others 2007; Furusyo and others 2007). According to the Th1/Th2 paradigm, inflammation associated with allergic disorders is regarded as a Th2-dominant immune response. The cytokines liberated by allergen-reactive Th2 cells control the process leading to allergic inflammation. Thus specific control of p63 expression may lead to a new modality for the control of allergic and atopic disorders, or even cancer, through alteration of the levels of TARC/CCL17 (Ishida and Ueda 2006; Saeki and Tamaki 2006).

Previously we have reported that p63 can also activate the gene expression of human inter-cellular adhesion molecule 1 (ICAM-1), which plays a role in the binding of T cells through lymphocyte function–associated antigen-1 (LFA-1)

to epithelial cells. This mechanism may further work to enforce the affinity of epithelial cells to T cells. The promoter region of the ICAM-1 gene responsive to p63 is restricted, with a 135-bp region where an interferon-γ-activated sequence (GAS) motif, Sp-1, and AP-2 are localized (Kikuchi and others 2004). It is noteworthy that this GAS motif is seemingly shared by the proximal promoter sequence of TARC/CCL17 (Maier and others 2007). However, within a promoter sequence from 950-bp to the transcription start site of the TARF/CCL17 promoter region, we failed to find a sequence that completely matched the specific motif directly bound to p63 reported recently (Perez and others 2007). Thus it is possible to consider that p63 indirectly modulates the transactivation of the TARC/CCL17 gene through GAS or other motifs via an as yet unidentified mechanism, although more experiments will be required to determine the precise action of p63 in the regulation of these genes.

In summary, we reported novel involvement of p63 in the regulation of TARC/CCL17 of epithelial tissues under tissue repair. To respond rapidly and effectively to various immunologic insults, the coordinated action of various resident cell populations around epithelial cells as well as immigrating leukocytes enables the mucosal immune system. Our results may also shed light on the role of p63 in epithelial immune surveillance in terms of maintaining peripheral T-cell tolerance as well (Sather and others 2007).

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Address reprint requests or correspondence to:
Shingo Ichimiya, M.D., Ph.D.
Department of Pathology
Sapporo Medical University School of Medicine
Sapporo 060-8557
Japan

Tel: +81-11-611-2111 (ext. 2691)
Fax: +81-11-643-2310
E-mail: ichimiya@sapmed.ac.jp

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Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas

M Murase[1,2,5], M Kano[1,2,5], T Tsukahara[*,1,2], A Takahashi[3], T Torigoe[2,3], S Kawaguchi[1], S Kimura[1], T Wada[1], Y Uchihashi[4], T Kondo[4], T Yamashita[1] and N Sato[2]

[1]Department of Orthopaedic Surgery, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo 060-8543, Japan; [2]Department of Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan; [3]Cancer Vaccine Laboratory, Japan Science and Technology Corporation, North-19, West-11, Kita-ku, Sapporo 060-0819, Japan; [4]Team for Cell Linage Modulation, RIKEN Center for Developmental Biology, 2-2-3, Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan

BACKGROUND: Several human cancers have been found to contain cancer stem-like cells (CSCs) having cancer-initiating ability. However, only a few reports have shown the existence of CSCs in bone and soft tissue sarcomas. In this study, we identified and characterised side population (SP) cells that showed drug-resistant features in human bone sarcoma cell lines.

METHODS: In seven osteosarcoma cell lines (OS2000, KIKU, NY, Huo9, HOS, U2OS and Saos2) and in one bone malignant fibrous histiocytoma (MFH) cell line (MFH2003), the frequency of SP cells was analysed. Tumourigenicity of SP cells was assessed *in vitro* and *in vivo*. Gene profiles of SP cells and other populations (main population; MP) of cells were characterised using cDNA microarrays.

RESULTS: SP cells were found in NY (0.31%) and MFH2003 (5.28%). SP cells of MFH2003 formed spherical colonies and re-populated into SP and MP cells. In an NOD/SCID mice xenograft model, 1×10^3 sorted SP cell-induced tumourigenesis. cDNA microarray analysis showed that 23 genes were upregulated in SP cells.

CONCLUSIONS: We showed that SP cells existed in bone sarcoma cell lines. SP cells of MFH2003 had cancer-initiating ability *in vitro* and *in vivo*. The gene profiles of SP cells could serve as candidate markers for CSCs in bone sarcomas.

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Over the past three decades, there have been remarkable advances in the treatment of bone and soft tissue sarcomas. These include the introduction of adjuvant chemotherapy, establishment of guidelines for adequate surgical margins and the development of post-excision reconstruction (Fletcher *et al*, 2002; Lewis, 2007). However, the prognosis of non-responders to chemotherapy is still poor and the mechanisms of tumourigenesis of bone and soft tissue sarcomas remain to be demonstrated.

Generally, cancer masses are considered to be a complex of heterogeneous but equally malignant cell populations. However, recent stem cell research on the development of normal organs has drawn attention to the existence of a 'cancer stem-like cell (CSC)' counterpart, which is characterised by its self-renewal capacity, differentiation potential, and cancer-initiating ability (Visvader and Lindeman, 2008). On the basis of these characteristics, CSCs have been postulated to be responsible for driving the growth of tumours and for the recurrence of neoplasms after current therapeutic modalities are used.

*Correspondence: Dr T Tsukahara, Department of Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan; E-mail: tukahara@sapmed.ac.jp
[5]These authors contributed equally to this work.
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Initial attempts to characterise CSCs were accomplished using cell surface molecules in acute myeloid leukaemia. Several groups that found CSCs capable of initiating leukaemia were found in the $CD34^+CD38^-$ fraction (Warner *et al*, 2004). Recently, CSCs have been isolated from several human solid tumours that have markers for putative normal stem cells, including breast cancer ($CD44^+CD24^-ESA^+$) (Al-Hajj *et al*, 2003), pancreatic cancer ($CD44^+CD24^-ESA^+$, $CD133^+CXCR4^+$) (Hermann *et al*, 2007; Li *et al*, 2007), brain cancer ($CD133^+$) (Singh *et al*, 2004), prostate cancer ($CD44^+/\alpha_2\beta_1^{hi}/CD133^+$) (Collins *et al*, 2005), hepatocellular carcinoma ($CD133^+$) (Yin *et al*, 2007) and colon cancer ($CD133^+$) (Ricci-Vitiani *et al*, 2007).

On the other hand, in the analysis of haematopoietic stem cells, a sub-population that effluxes the DNA-binding dye Hoechst 33342 out of the cell membrane through an ATP-binding cassette (ABC) transporter was recognised as a stem cell population (Goodell *et al*, 1996; Zhou *et al*, 2002; Robinson *et al*, 2005). This cell population expressing the ABC transporter was defined as side population (SP) cells, which were distinguished from cells of the other population (main population; MP). Recent studies demonstrated that SP cells could be characterised as CSCs in primary tissues of gastrointestinal cancers (Haraguchi *et al*, 2006) and ovarian cancer (Szotek *et al*, 2006). SP cells were also shown in established tumour cell lines with different origins, such as glioma (Kondo *et al*, 2004), breast (Kruger *et al*, 2006) and thyroid cancer monoclonal cell lines (Mitsutake *et al*, 2007).

To date, however, distinct molecular markers on CSCs are still lacking in many cancers. Moreover, only a few reports have shown the existence of CSCs in bone and soft tissue sarcomas (Gibbs *et al*, 2005; Wu *et al*, 2007; Tirino *et al*, 2008). In this study, with the goal of determining specific markers of CSCs, we identified and characterised SP cells having cancer-initiating ability in osteosarcoma and bone malignant fibrous histiocytoma cell lines.

MATERIALS AND METHODS

Cell lines and culture

Seven human osteosarcoma (OS) cell lines (OS2000, KIKU, NY, Huo9, HOS, U-2OS and Saos2) and one bone human malignant fibrous histiocytoma (MFH) cell line (MFH2003) were used. OS2000, KIKU and MFH2003 were established in our laboratory (Wada *et al*, 1988; Nabeta *et al*, 2003; Tsukahara *et al*, 2006). The other cell lines were kindly donated by or purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) and from the American Type Culture Collection (Manassas, VA, USA). MFH2003 and OS2000 were cultured with Iscove's modified Dulbecco's Eagle's medium (IMDM; GIBCO BRL, Grand Island, NY, USA) containing 10% FBS and the others were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) containing 10% FBS in a 5% CO_2 incubator at 37°C.

Identification of side population

Cell suspensions were labelled with dye Hoechst 33342 (Cambrex Bio Science Walkersville Inc., MD, USA) using the methods described by Goodell *et al* (1996) with some modifications . Briefly, cells were trypsinised and re-suspended in pre-warmed DMEM supplemented with 5% FBS at a concentration of $1 \times 10^6\,ml^{-1}$. Hoechst33342 dye was added at a final concentration of 2.5 or $5.0\,\mu g\,ml^{-1}$ in the presence or absence of verapamil (50 or 75 μM; Sigma-Aldrich) as an inhibitor of the ABC transporter. The cells were incubated at 37°C for 90 min with continuous shaking. At the end of the incubation, the cells were washed with ice-cold PBS with 5% FBS, centrifuged at 4°C and resuspended in ice-cold PBS containing 5% FBS. Propidium iodide (at a final concentration of $1\,\mu g\,ml^{-1}$; Molecular Probes–Invitrogen, Eugene, OR, USA) was used to gate viable cells. Flow cytometry and cell sorting were performed using FACSVantage SE (BD Biosciences, Bedford, MA, USA), EPICS ALTRA (Beckman-Coulter, Fullerton, CA, USA) and FACS Aria II (BD Biosciences). The Hoechst 33342 dye was excited at 357 nm and its fluorescence was analysed using dual wavelengths (blue, 402–446 nm; red, 650–670 nm).

RNA preparation

Total RNAs were extracted from SP cells and MP cells using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Using an Amino Allyl MessageAmp aRNA Kit Ver. 2 (Sigma-Aldrich Japan, Ishikari, Japan), amino allyl-modified aRNAs were prepared from total RNAs from SP and MP cells as previously described (Takeuchi *et al*, 2008).

Real-time PCR analysis

Total RNA was reverse transcribed using the SuperScriptIII reverse transcriptase enzyme (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with SYBR Green Real-time Core Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Primers were designed to generate a PCR product of <200 bp. The thermal cycling conditions were 94°C for 2 min, followed by 35 cycles of 15 s at 94°C, 30 s at 60°C and 30 s at 72°C. Levels of expression were normalised to the *glyceraldehyde-3-phosphate dehydrogenase* (G3PDH) housekeeping gene.

Spherical colony formation assay

Spherical colony formation assay was performed as described by Gibbs *et al* (2005) with some modifications. Briefly, cells were plated at 2000 cells per well in six-well ultra-low attachment plates (Corning Inc., Corning, NY, USA). Mesenchymal Stem Cell Basal Medium (MSCBM) and MSCBM SingleQuots (Takara Bio Inc., Ohtsu, Japan) were used for cell culture. Fresh aliquots of epidermal growth factor and basic fibroblast growth factor were added every other day. On day 14, the numbers of colonies were counted.

Xenograft model

Sorted SP and MP cells of MFH2003 were collected and re-suspended at 1×10^2–1×10^5 cells per 50 μl of PBS, followed by addition of 50 μl of Matrigel (BD Biosciences). This cell-Matrigel suspension was subcutaneously injected into the backs of 4- to 6-week-old non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.CB17-Prdkc^{scid}/J, Charles River Laboratory, Yokohama, Japan) under anaesthesia. Mice were observed for up to 12 weeks.

Gene expression profiling using cDNA microarrays

The aRNAs from SP cells were labelled with Cy5 dye and those from MP cells were labelled with Cy3 dye. The dye-labelled aRNA samples were hybridised to a 29 138-spot Human Panorama Micro Array (Sigma-Aldrich) for 16 h at 45°C. The intensities of Cy5 and Cy3 fluorescence for every gene spot on the hybridised array were measured with a GenePix 4000B scanner (Axon Instrument, Austin, TX, USA), and were analysed with GenePix Pro 5.0 software (Axon Instrument). Global normalisation of the resultant data was carried out using Excel 2004 (Microsoft, Redmond, WA, USA). As a result, 24 730 genes were available for further analysis. The average expression ratio of Cy5 to Cy3 was obtained for each gene. A dye-swap experiment (labelling SP and MP cells with Cy3 and Cy5, respectively) was also performed. An average ratio of more than 2.0, reproducible in two experiments, was determined to indicate differential upregulation in SP cells.

RESULTS

Identification of SP cells in human bone sarcoma cell lines

To identify the CSCs of bone sarcomas, we tried to detect side population (SP) cells in bone and soft tissue sarcoma cell lines. As depicted in Figure 1A, the NY and MFH2003 cell lines included 0.38 and 5.28% SP cells, respectively. In each case, the percentage of SP cells was markedly diminished by treatment with verapamil, which is an inhibitor of the pumps responsible for the exclusion of Hoechst dye, indicating that this population truly represented SP cells. SP cells were hardly detected in the other cell lines (Figure 1B). Therefore, MFH2003, containing the highest proportion of SP cells, was selected and further analysed.

Isolation of SP cells and their repopulation as both SP and MP cells

After excluding dead cells and cellular debris on the basis of scatter signals and propidium iodide fluorescence, MFH2003 cells were sorted into SP and MP cells. As shown in Figure 2A, the G1 gate showed the SP cells that had negative/low patterns of staining with Hoechst 33342, and the G2 gate showed the main population cells that were positively stained with Hoechst 33342. To ascertain the

purity of sorted cells, the obtained SP (G1) cells and MP (G2) cells were re-analysed. The purity levels were 92.86 and 90.78% in the SP population and MP population, respectively. These results supported the specificity for further analysis using the resultant SP and MP cells.

To examine whether SP cells could generate both SP and MP cells, sorted SP and MP cells were further cultured *in vitro*. On day 14, the cells were re-stained with Hoechst 33342 and analysed by flow cytometry. We observed that SP cells re-populated both SP and MP cells. The ratio of SP cells to MP cells was still much higher than that before sorting. In contrast, SP cells were not detected in sorted MP cells.

Expression of *ABCG2* mRNA, which is a marker of SP cells, was increased in SP cells (Figure 2B). These results also supported the specificity for further characterisation of SP cells, especially with regard to their cancer-initiating ability.

Spherical colony formation

We next evaluated the ability of SP and MP cells to generate spherical colonies. A total of 2000 SP and MP cells were sorted and cultured immediately under conditions of serum starvation, providing an anchorage-independent environment. On day 14, SP cells showed spherical colony formation (Figure 3A). On the

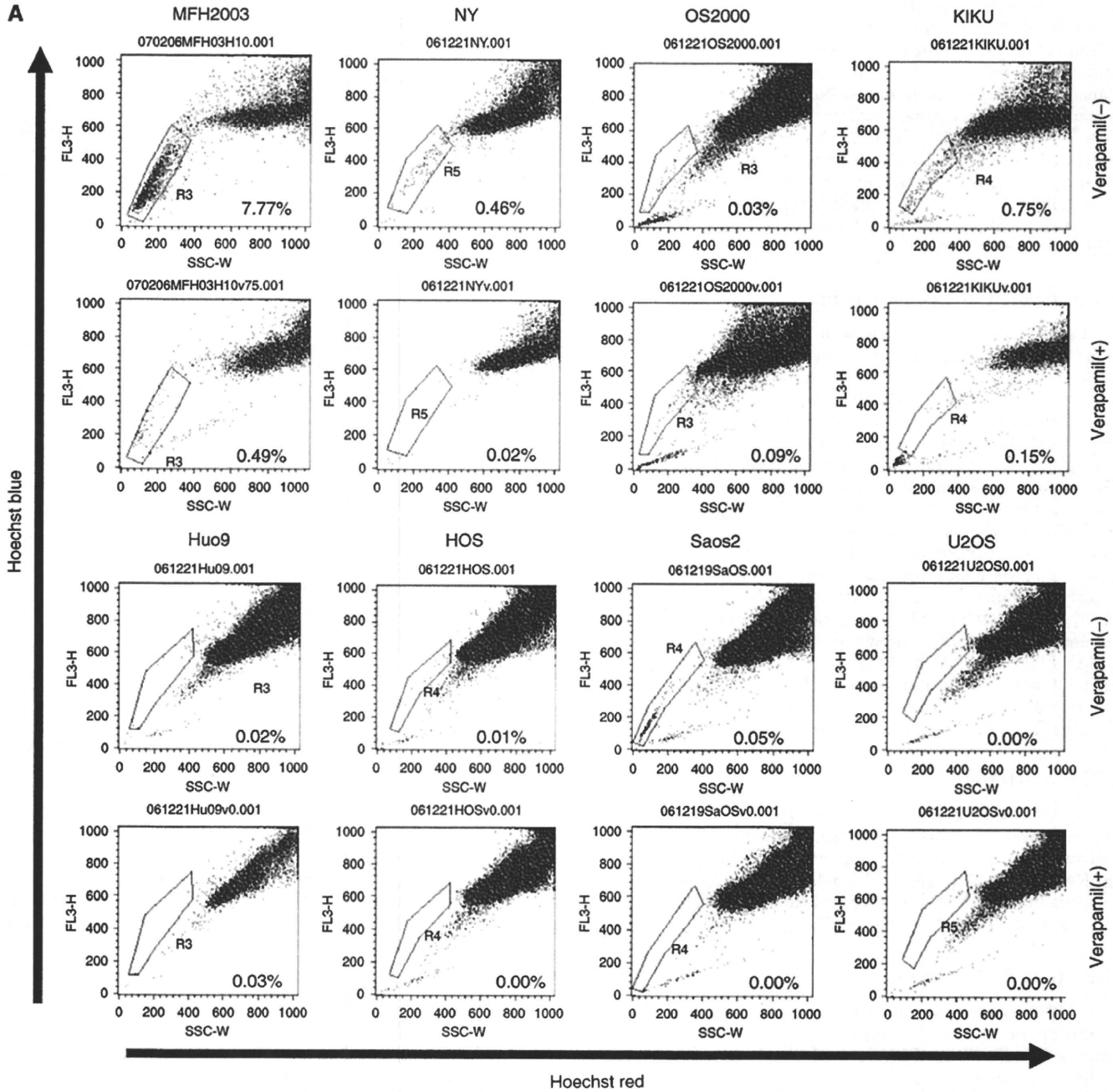

Figure 1 Detection of side population cells in bone sarcoma cell lines. (**A**) The populations of SP cells of seven osteosarcoma cell lines (NY, OS2000, KIKU, Huo9, HOS, Saos2 and U2OS) and of one bone MFH cell line (MFH2003), in the presence or absence of verapamil, are shown. SP cells are marked by black dotted lines to show the proportion of SP cells among total living cells. (**B**) The mean proportions of SP cells in cell lines. These results were reproducible in at least two independent experiments.

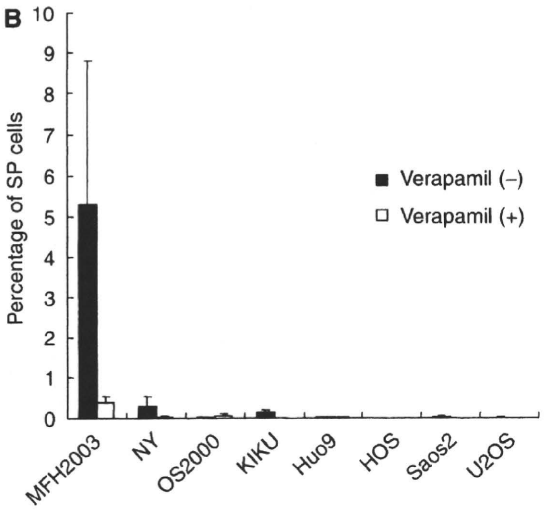

Figure 1 Continued.

other hand, as shown in Figure 3B, most MP cells died and the others formed a few small colonies. We removed spherical colonies from the suspension culture and attempted again to determine whether the cells could attach to a substratum. As shown in Figure 3C, cells were seen expanding from the sphere. In Figure 3D, the number of colonies is shown, indicating clearly that, among MFH2003 cells, SP, but not MP, cells had the potential for spherical colony formation.

Cancer-initiating ability of SP and MP cells *in vivo*

To address the issue of whether tumourigenic activity differed between SP and MP cells, $1 \times 10^2 - 1 \times 10^5$ SP and MP cells sorted from MFH2003 were injected into NOD/SCID mice (Figure 4A). To rule out the effects of the toxicity of Hoechst, we routinely performed (i) depletion of dead cells by PI staining and (ii) a viability check using trypan-blue staining after cell sorting. Almost all MP cells were viable as SP cells. Subcutaneous tumour formation was induced by the injection of 1×10^3 SP cells (Table 1). We also observed that 1×10^4 SP cells formed a larger tumour mass than did 1×10^3 SP cells (data not shown). In contrast, at least 1×10^5 MP cells were required to give rise to a tumour. Macroscopic and microscopic findings of a tumour derived from SP cells are shown in Figure 4A and B. These results supported the hypothesis that SP cells have a high cancer-initiating ability, similar to CSCs. At 8 weeks after xenotransplantation, the frequencies of SP and MP cells in a formed tumour derived from 1×10^4 SP cells were analysed *ex vivo*. SP cells were hardly detected in the tumour. Most SP cells re-populated into MP cells *in vivo* in 8 weeks (data not shown).

Identification of upregulated genes in SP cells

Finally, we performed gene expression profiling of SP cells using cDNA microarrays. As shown in Table 2, 23 genes were found to be upregulated in SP cells, compared with MP cells. Although the functions of upregulated genes varied, these results suggested that the factors connected to DNA transcription, transport of substrates, cell proliferation and apoptosis might have a role in the cancer-initiating ability of SP cells. In addition, the increased expression of *ABCG2* in SP cells confirmed the accuracy of the gene expression profiling analysis.

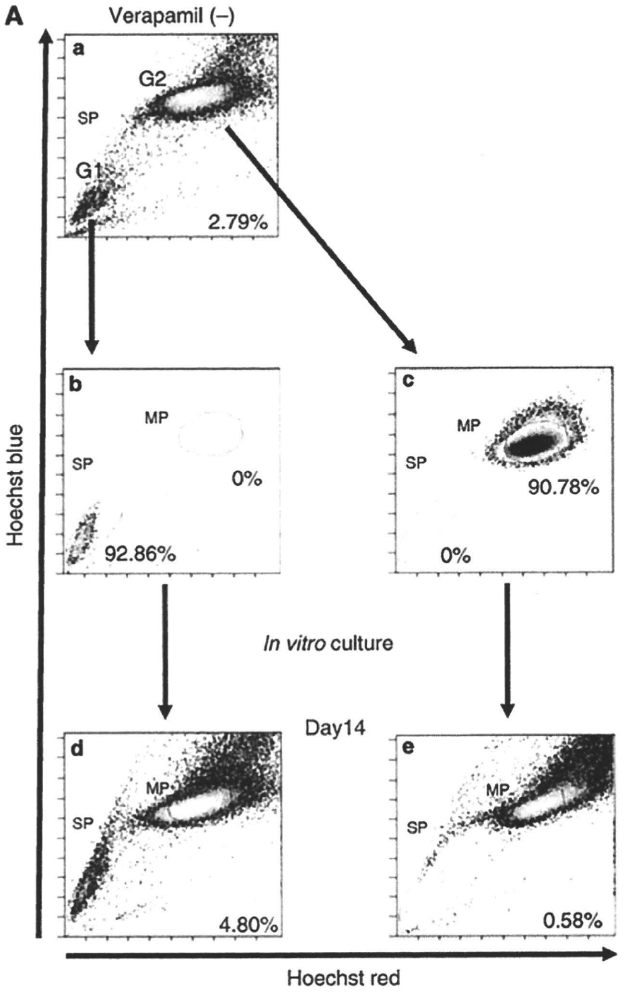

Figure 2 The re-population of SP cells into both SP and MP cells. (**A**) (**a**) The populations of SP cells and MP cells before cell sorting are shown. SP cells were gated as G1, and MP cells were gated as G2. (**b**, **c**) The proportions of SP cells among the total living cells are indicated. Isolated SP cells (**b**) and MP cells (**c**) after cell sorting. The proportions of SP and MP cells among the total living cells are indicated. (**d**, **e**) The populations of SP cells (**d**) and MP cells (**e**) after 2-week *in vitro* culture with medium containing 10% FBS are also shown. Experiments were repeated in triplicate with similar results. (**B**) The relative expression of ABCG2 was evaluated in SP cells and MP cells by real-time PCR.

DISCUSSION

In this study, we showed that (i) small SP populations existed in one osteosarcoma cell line and one bone MFH cell line; (ii) SP cells derived from MFH2003 could re-populate both SP and MP cells *in vitro*; (iii) SP cells could form spherical colonies and re-populate into SP and MP cells; (iv) SP cells had tumourigenesis in an *in vivo* xenograft model; and (v) factors regarding transcription, cell proliferation and apoptosis were upregulated in SP cells.

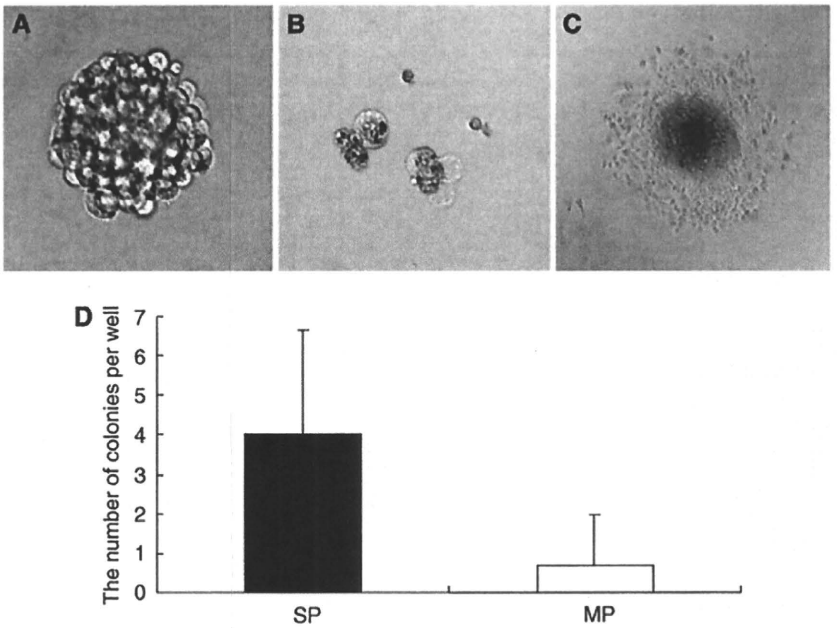

Figure 3 Tumourigenesis of SP and MP cells *in vitro*. (**A** and **B**) The features of spherical colonies derived from resultant SP cells (**A**) and MP cells (**B**) cultured without serum in an anchorage-independent manner for 2 weeks. (**C**) Spherical colony removed from the suspension culture and allowed to attach to a substratum. Adherent cells can be seen expanding from the sphere. (**D**) The numbers of resultant spherical colonies from SP cells and MP cells were counted. Data are representative of three independent experiments.

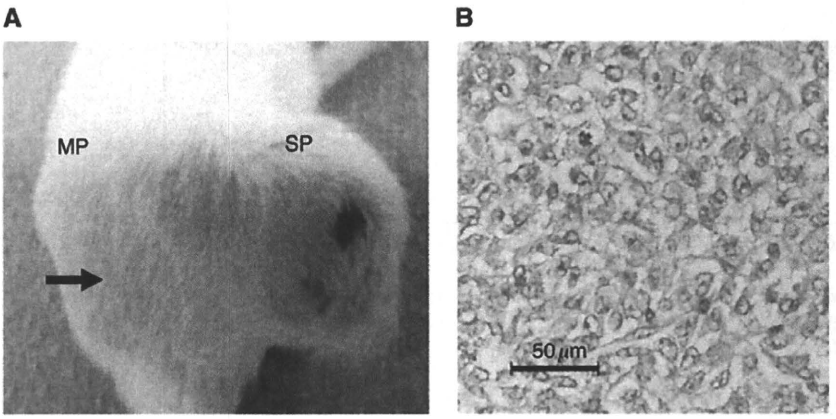

Figure 4 The features of xenotransplanted SP cells *in vivo*. (**A**) Macroscopic features of 1×10^3 each of SP and MP cells in an NOD/SCID mouse at 12 weeks after xenotransplantation. Black arrow indicates the site of injection of MP cells. (**B**) Histological findings of the xenotransplanted tumour derived from SP cells (1×10^4). Haematoxilin and eosin staining (original magnification: × 200) is shown.

Table 1 Tumorigenesis of SP and MP cells in NOD/SCID mice

	Cell number for injection			
	1×10^2	1×10^3	1×10^4	1×10^5
MFH2003				
SP cells	0/5	1/5	2/5	1/2
MP cells	0/5	0/5	0/5	1/2

SP and MP cells were isolated separately and injected into the backs of the subcutaneous space of NOD/SCID mice. Tumour formation was observed for 12 weeks after injection.

We observed proportions of SP cells of 0.31 and 5.28% in NY and MFH2003, respectively. The proportions of SP cells we observed were similar to those in most previous reports, with 2% noted in human breast cancer cell line MCF7, 0.4% in rat C6 glioma, 1.2% in human HeLa carcinoma (Kondo *et al*, 2004) and 4–37% in neuroblastoma cell lines (Hirschmann-Jax *et al*, 2004).

The SP cells were defined by the efflux of Hoechst 33342, a cell-permeable DNA-specific bisbenzimidazole dye, through an ABC transporter. Therefore, SP cells are considered to be resistant to multi-chemotherapeutic drugs and to confer malignant phenotypes to tumours (Dean *et al*, 2005). Hence, the characterisation of SP cells might be a useful tool for analysis of CSCs, especially when specific CSC surface markers are unknown.

We found that SP cells could re-populate both SP cells and MP cells *in vitro*. These results suggested that SP cells were capable of self-renewal and also generated MP cells by asymmetric division. This indicated that a tumour hierarchy might exist in bone MFH. Previous studies have also shown that SP cells can divide asymmetrically and display a capacity for self-renewal similar to

Table 2 List of genes upregulated in SP cells of MFH2003

Gene symbol	Gene name	Accession no.	Gene ontology	Expression ratio (SP/MP) Cy5/Cy3	Cy3/Cy5
ANKRD11	Ankyrin repeat domain 11	NM_013275	Electron transport	2.1	2.7
SLC2A4	Solute carrier family 2, member 4	NM_001042	Carbohydrate transport	2.2	2.5
KIAA1440	KIAA1440	AB037861	Unknown	2.2	3.3
SURF6	Surfeit 6	NM_006753	Ribosome biogenesis	2.3	2.8
VPF	Vascular permeability factor	M27281	Cell proliferation	3.0	3.5
C20orf14	Chromosome 20 open reading frame 14	NM_012469	RNA processing	2.1	3.6
PHLDA3	Pleckstrin homology-like domain, family A, member 3	NM_012396	Physiological processes	2.1	2.4
ZNF19	Zinc finger protein 19	NM_152907	Regulation of transcription	2.8	2.4
MCL1	Myeloid cell leukaemia sequence 1	NM_021960	Apoptosis	2.1	3.9
APOE	Apolipoprotein E	NM_000041	Lipid transport	2.1	3.8
NR4A2	Nuclear receptor subfamily 4, group A, member 2	NM_006186	Regulation of transcription	2.5	4.1
IRX3	Iroquois-related homeobox 3	BC023667	Regulation of transcription	3.3	2.9
GNB3	Guanine nucleotide-binding protein, β-polypeptide 3	NM_002075	G-protein coupled receptor protein signaling	2.1	5.0
NLRP12	NLR family, pyrin domain containing 12	NM_144687	Apoptosis	2.1	2.3
PTN	Pleiotrophin	NM_002825	Neurogenesis	2.0	2.6
ABCG2	ATP-binding cassette, sub-family G, member 2	NM_004827	Transport	2.2	2.9
APOL1	Apolipoprotein L	NM_145343	Lipid transport	2.2	2.6
MDFI	MyoD family inhibitor	NM_005586	Unknown	2.8	3.1
PRSS15	Protease, serine, 15	NM_004793	ATP-dependent proteolysis	2.1	2.5
MSX1	Msh homeo box homolog 1	NM_002448	Regulation of transcription	2.1	2.8
LDLR	Low density lipoprotein receptor	NM_000527	Cholesterol metabolism	2.1	3.1
LMNA	Lamin A/C	NM_170707	Cellular morphogenesis	2.1	2.9
MVK	Mevalonate kinase	BC016140	Cholesterol biosynthesis	2.2	2.1

Genes showing the ratio more than 2.0, which were reproducible in two experiments, were listed.

normal stem cells (Kondo *et al*, 2004; Singh *et al*, 2004). On the other hand, we also observed that most SP cells xenotransplanted in NOD/SCID mice re-populated into MP cells *in vivo*. SP cells might hardly be maintained *in vivo* for long time, that is, more than 8 weeks. In other words, the niche of the mouse model might not be adequate for the maintenance of SP cells derived from bone sarcoma cell lines, such as MFH2003.

The ability of SP cells to generate spherical colonies was higher than that of MP cells. This is consistent with previous studies (Patrawala *et al*, 2005; Mitsutake *et al*, 2007). We recognised that the difference was not a consequence of longer retention of Hoechst dye in MP cells, because MP cells were viable after staining with the dye, followed by sorting and maintenance in a culture medium with FBS. However, we cannot completely rule out the possibility that the difference was due to some effect of the Hoechst dye, which is potentially cytotoxic (Durand and Olive, 1982).

We could detect a higher tumourigenic potential of SP cells than of MP cells *in vivo* using a NOD/SCID xenograft model. In the field of bone and soft tissue sarcoma, only Wu *et al* (2007) succeeded in showing the *in vivo* cancer-initiating ability of CSCs derived from bone and soft tissue sarcomas, using SP cells isolated from fresh primary tumour tissues. The ability to consistently isolate MFH2003-derived SP cells allowed us to conduct SP cell-specific gene profiling. Moreover, it might become possible to identify CTL-defined CSC-specific tumour antigens for immunotherapy targeting CSCs. Thus far, we have been trying to identify CTL-defined tumour antigens by forward and reverse immunological approaches and have carried out antigenic peptide vaccination trials in bone and soft tissue sarcomas (Sato *et al*, 2002; Ida *et al*, 2004; Tsukahara *et al*, 2004, 2008a, b, 2009; Kawaguchi *et al*, 2005; Kimura *et al*, 2008). Currently, we are trying to establish autologous CTL clones recognising SP cells of MFH2003 from tumour-infiltrating lymphocytes.

Thus far, only Oct3/4, Nanog and CD133 were reported to be candidates for CSC-specific markers in bone and soft tissue sarcoma (Gibbs *et al*, 2005; Tirino *et al*, 2008). Therefore, the gene profile of SP cells might help to expand the possibility of an effective isolation of CSCs from bone and soft tissue sarcomas using these specific markers. In the current gene expression profiling, 23 genes with various functions were upregulated in SP cells. Among them, eight genes (*VPF*, *c20orf14*, *MCL1*, *NR4A2*, *IRX3*, *NRLP12*, *PTN* and *LMNA*) might be considered to be potential tumourigenic factors in malignancies. VPF, generally known as vascular endothelial growth factor, regulates vascular permeability, angiogenesis, cell migration and apoptosis in tumours (Nagy *et al*, 2008). C20orf14 is upregulated in lymphoma (Su *et al*, 2008) and HPV16/18-positive cervical cancer (Vazquez-Ortiz *et al*, 2007). MCL1 is a member of the B-cell lymphoma (BCL) family. MCL-1 negatively regulates pro-apoptotic factors (Bak and Bax) (Chen *et al*, 2007) and accelerates leukaemogenesis (Beverly and Varmus, 2009). NR4A2 belongs to the steroid nuclear hormone receptor superfamily and has a role in cell transformation in cervical cancer (Ke *et al*, 2004). IRX3 is epigenetically inactivated by methylation in CpG islands in brain tumours (Ordway *et al*, 2006) and prostate cancer (Morey *et al*, 2006). NLRP12, also known as RNO2/monarch-1, is reported to activate inflammation in humans (Ye *et al*, 2008). PTN is an angiogenic factor that stimulates tumour-associated vascular formation in many malignancies. (Perez-Pinera *et al*, 2008). LMNA is reported to encode lamin A, which is a putative colonic epithelial stem cell marker and is also a prognostic factor in colorectal cancer (Willis *et al*, 2008). On the other hand, four genes (*ANKRD11*, *PHLDA3*, *APOL1* and *MSX1*) are known as tumour-suppressor factors. ANKRD11 is a p53-interacting protein and activates the transcription of p53 in breast cancer. PHLDA3 is a positive regulator of Fas-dependent death signalling, related to cisplatin-mediated apoptosis (Kerley-Hamilton *et al*, 2005). APOL1 is classically thought to be involved in lipid transport and metabolism and has rarely been characterised with regard to cell survival. Although the structure of APOL1 is similar to that of the anti-apoptotic proteins of the Bcl-2 family (Vanhollebeke and Pays, 2006), it can induce autophagic cell death (Wan *et al*, 2008). MSX1, a homeobox gene important for embryonic neural crest development, can induce the inhibition of tumour-initiating ability in soft agar *in vitro*. Taken together, the gene expression profiling in SP cells derived from MFH2003 containing various tumour-proliferative and tumour-suppressive factors might indicate the complexity of maintaining the

characteristics of SP cells as tumour initiators. However, considering the characteristics of SP cells for cancer-initiating ability, the apoptosis-related molecules among these genes (MCL-1, ANKRD11, PHLDA3 and APOL1) might have roles in the proliferation of SP cells. Moreover, these molecules could be candidates for specific markers and, in addition, molecular therapeutic targets.

In conclusion, we identified SP cells in established human bone sarcoma cell lines. Moreover, we demonstrated that bone MFH-derived SP cells can re-populate both SP and MP cells and have cancer-initiating ability *in vitro* and *in vivo*. These findings supported the idea that bone sarcomas might contain a certain population of CSCs. Gene profiling of SP cells could serve to elucidate candidates for specific markers and therapeutic targets. Thus, further studies for the characterisation of CSCs in human bone and soft tissue sarcomas might contribute to the elucidation of the mechanisms of tumourigenesis and to the establishment of novel therapeutic strategies.

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Targeting to Static Endosome Is Required for Efficient Cross-Presentation of Endoplasmic Reticulum-Resident Oxygen-Regulated Protein 150-Peptide Complexes[1]

Goro Kutomi,*† Yasuaki Tamura,[2]* Koichi Okuya,*† Takashi Yamamoto,* Yoshihiko Hirohashi,* Kenjiro Kamiguchi,* Jun Oura,* Keita Saito,*† Toshihiko Torigoe,* Satoshi Ogawa,‡ Koichi Hirata,† and Noriyuki Sato*

Heat shock proteins (HSPs) such as Hsp70, gp96, and Hsp90 have been shown to elicit intriguing, efficient CTL responses by cross-presentation via an as yet entirely unknown mechanism. Oxygen-regulated protein 150 (ORP150), also known as grp170, is an endoplasmic reticulum-resident HSP and is up-regulated by hypoxia. It has been demonstrated that ORP150 binds tumor-associated Ag peptides within cancer cells. Immunization with an ORP150-tumor Ag complex has been shown to generate tumor-specific CTLs. Most recently, it has been shown that exogenous ORP150 induces cross-presentation of a chaperoned Ag, thereby stimulating Ag-specific CTLs. However, the mechanism underlying this efficient cross-presentation is still unsolved. In this study, we show that the ORP150-precursor peptide complex can elicit CTL response through cross-presentation as well as the CD4$^+$ T cell response by dendritic cells. Furthermore, we observed that the internalized ORP150-peptide complex, but not OVA protein, which was not cross-presented, was sorted to the Rab5$^+$, EEA1$^+$ static early endosome, followed by translocation to a recycling endosome, where the ORP150-chaperoned peptide was processed and bound to MHC class I molecules. Moreover, we observed that immunization of mice with ORP150-peptide complexes elicited strong peptide-specific CTLs and antitumor effects in vivo. Our data indicate that targeting of the Ag to a "static" early endosme by ORP150 is required for the efficient cross-presentation. *The Journal of Immunology,* **2009, 183: 5861–5869.**

It is well known that tumor-derived heat shock proteins (HSPs)[3] such as Hsp70, Hsp90, and gp96 initiate efficient tumor-specific CTL responses and protective immunity (1–5). Although immunized HSPs are exogenous Ags, these HSP-Ag complexes can gain access to the class I Ag presentation pathway, resulting in the stimulation of CD8$^+$ T cells, termed cross-presentation (6–11). The ability of HSPs to facilitate the cross-presentation of MHC class I-restricted epitopes and to prime CD8$^+$ T cell responses is due to the following: (1) HSPs are able to form stable complexes with antigenic peptides/proteins, (2) HSP-peptide/protein complexes are able to bind surface receptors on APCs, resulting in receptor-dependent endocytosis, and (3) HSP can stimulate an innate immune response, which is not dependent on tumor Ags (12, 13). It is thought that HSPs bind to receptors on APCs, resulting in secretion of proinflammatory cytokines and maturation and activation of dendritic cells (DCs). To date, CD91 (14–16), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (17), CD40 (18), scavenger receptor type A (SR-A) (19, 20), and scavenger receptor expressed by endothelial cells-I (SREC-I) (20, 21) have been demonstrated to be receptors for several kinds of HSPs expressed on APCs.

DCs are the most potent APCs for efficient cross-presentation. Recently, it has been shown that DCs can internalize HSP-peptide complexes by receptor-mediated endocytosis and direct chaperoned peptides into the intracellular pathway for MHC class I-restricted presentation to CD8$^+$ T cells, concomitant with the induction of dendritic cell maturation and cytokine secretion (10). However, the underlying mechanism for efficient cross-presentation, in particular how the HSP-Ag complex can enter the MHC class I pathway, is not well understood. Recently, we have demonstrated that extracellular Hsp90-peptide complexes are efficiently cross-presented via the endosome-recycling pathway (22). In this Hsp90-mediated cross-presentation, the receptor-dependent endocytosed Hsp90-peptide complex was transferred to the early endosome in which a cysteine protease such as cathepsin S processed the precursor peptide. The resulting MHC class I epitope was transferred onto recycling MHC class I molecules, thereby expressing an MHC class I-epitope complex on the cell surface. Furthermore, we have shown that immunization with Hsp90-tumor Ag peptide complexes induces Ag-specific CTL responses and strong antitumor immunity in vivo.

Oxygen-regulated protein 150 (ORP150), also known as glucose-regulated protein 170 (grp170), was first described in 1996 by Kuwabara et al. (23). It is an endoplasmic reticulum (ER)-resident Hsp70 superfamily member, and it is induced by stress conditions such as hypoxia, ischemia, glucose deprivation, reductive reagents,

*Department of Pathology and †Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan; and ‡Department of Neuroanatomy, Kanazawa University Medical School, Kanazawa, Ishikawa, Japan

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[2] Address correspondence and reprint requests to Dr. Yasuaki Tamura, Department of Pathology, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan. E-mail address: ytamura@sapmed.ac.jp

[3] Abbreviations used in this paper: HSP, heat shock protein; BMDC, bone marrow-derived dendritic cell; CPRG, chlorophenol red-β-D-galactopyranoside; DC, dendritic cell; ER, endoplasmic reticulum; HPF, high-power field; LAMP-1, lysosome-associated membrane protein 1; ORP150, oxygen-regulated protein 150; SR-A, scavenger receptor type A; SREC-I, scavenger receptor expressed by endothelial cells-I.

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