IFN-β

Fig.7 IFN-β mRNA levels in PH5CH8 cells transfected with LNA122-DS containing different length of dsRNA

PH5CH8 cells were transfected with LNA122-S containing different length of dsRNA. Total RNA was isolated from the cells 12 hrs after transfection. Expression levels of IFN-β mRNA were determined by quantitative RT-PCR. GAPDH mRNA levels were measured as an internal control. IFN-β mRNA level in the mock-transfected cells were normalized to 1. The data are expressed as means ± S.D. (n=4).
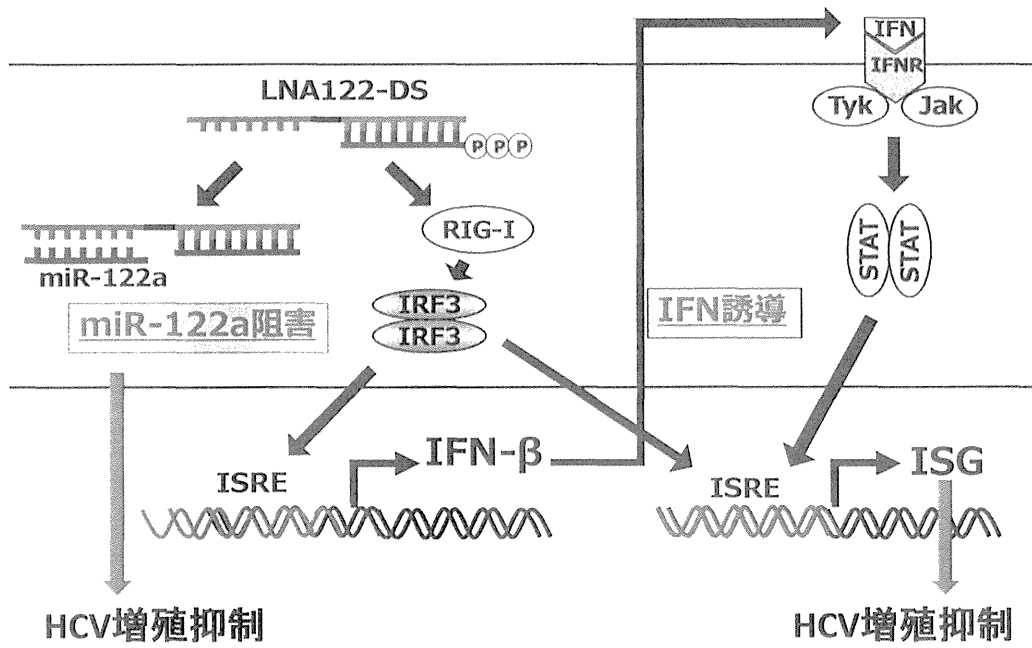
Figure 8. LNA122-DS-mediated suppression of HCV replication. IRF; IFN regulatory factor, ISRE; IFN-sensitive response element, ISG; IFN-stimulated gene, IFNR; IFN receptor, STAT; signal transducer and activator of transcription.

Table 1. Sequences of oligodeoxynucleotides used as a template for *in vitro* transcription of 3pssRNA.

Oligodeoxynucleotides	Sequence
ODN(+)-23bp-3pssRNA	5'-TAATACCTCACTATAGGGACACACACACACACACACAC-3'
ODN(+)-19bp-3pssRNA	5'-TAATACCTCACTATAGGGACACACACACACAC-3'
ODN(+)-21bp-3pssRNA	5'- TAATACCTCACTATAGGGACACACACACACACAC-3'
ODN(+)-25bp-3pssRNA	5'- TAATACCTCACTATAGGGACACACACACACACACACACAC-3'

Table 2. Sequences of primers for real-time RT-PCR.

Primers	Sequence
GAPDH-F	5'-GGTGGTCTCCTCTGACTTCAACA-3'
GAPDH-R	5'-GTTGCTGTAGCCAAATTCGTTGT-3'
IFN-B-F	5'-ATGACCAACAAGTGTCTCCTCC-3'
IFN-B-R	5'-GCTCATGGAAAGAGCTGTAGTG-3'
HCV-5'UTR-F	5'-GAGTGTCGTGCAGCCTCCA-3'
HCV-5'UTR-R	5'-CACTCGCAAGCACCCTATCA-3'
IFI6-F	5'-GGTCTGCGATCCTGAATGGG-3'
IFI6-R	5'-TCACTATCGAGATACTTGTGGGT-3'
ISG15-F	5'-GAACAAATGCGACGAACCTCT-3'
ISG15-R	5'-CCCTCGAAGGTCAGCCAGA-3'
ISG56-F	5'-CTTGAGCCTCCTTGGGTTCG-3'
ISG56-R	5'-GCTGATATCTGGGTGCCTAAGG-3'
OAS-F	5'-TGTCCAAGGTGGTAAAGGGTG-3'
OAS-R	5'-CCGGCGATTTAACTGATCCTG-3'

別紙4

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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櫻井文教、近藤昌夫	ウイルスを利用した医療・創薬研究の新展開-ウイルスは貴重なバイオマテリアル-	*Yakugaku Zasshi,*	133	289	2013

Two-Step Differentiation of Mast Cells from Induced Pluripotent Stem Cells

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Mast cells play important roles in the pathogenesis of allergic diseases. They are generally classified into 2 phenotypically distinct populations: connective tissue-type mast cells (CTMCs) and mucosal-type mast cells (MMCs). The number of mast cells that can be obtained from tissues is limited, making it difficult to study the function of mast cells. Here, we report the generation and characterization of CTMC-like mast cells derived from mouse induced pluripotent stem (iPS) cells. iPS cell-derived mast cells (iPSMCs) were generated by the OP9 coculture method or embryoid body formation method. The number of Safranin O-positive cells, expression levels of CD81 protein and histidine decarboxylase mRNA, and protease activities were elevated in the iPSMCs differentiated by both methods as compared with those in bone marrow-derived mast cells (BMMCs). Electron microscopic analysis revealed that iPSMCs contained more granules than BMMCs. Degranulation was induced in iPSMCs after stimulation with cationic secretagogues or vancomycin. In addition, iPSMCs had the ability to respond to stimulation with the IgE/antigen complex in vitro and in vivo. Moreover, when iPSMCs generated on OP9 cells were cocultured with Swiss 3T3 fibroblasts, protease activities as maturation index were more elevated, demonstrating that mature mast cells were differentiated from iPS cells. iPSMCs can be used as an in vitro model of CTMCs to investigate their functions.

Introduction

MAST CELLS HAVE RECENTLY gained attention, because they have been recognized as effector cells not only in allergic disorders, but also in other immune diseases, including autoimmune diseases and chronic inflammatory disorders [1]. Activation of mast cells triggers allergic and inflammatory responses through the release of a wide variety of mediators, such as histamine, arachidonic acid metabolites, and neutral proteases, and regulates immune responses through the production of cytokines and chemokines [2]. Mast cell precursors leave the bone marrow, migrate in the blood, invade tissues, and then proliferate and differentiate into mature cells [3]. Mature rodent mast cells are generally classified into 2 phenotypically distinct populations: connective tissue-type mast cells (CTMCs) and mucosal-type mast cells (MMCs) [3–4]. Each cell type differs with respect to location, staining characteristics, and histamine content. Mouse CTMCs, which are present in the peritoneal cavity and skin, contain heparin and store large amounts of histamine. In contrast, mouse MMCs, which are prominent in the mucosal layer of the gastrointestinal tract, contain chondroitin sulfate E rather than heparin and have relatively low histamine content. Since recent studies have demonstrated that CTMCs are involved in a wide variety of immune responses [5–7], development of an in vitro culture system of CTMCs is needed. Although several mast cell lines and IL-3-dependent bone marrow-derived mast cells (BMMCs) have been used as models to investigate the process of mast cell activation and subsequent production of proinflammatory mediators, these models have limitations in analyzing the functions specific to mature mast cells. Previous studies showed that coculture of BMMCs with Swiss 3T3 fibroblasts in the presence of stem cell factor (SCF) facilitated morphological and functional maturation toward a CTMC-like phenotype [8].

Differentiation of both mouse and human embryonic stem (ES) cells into multiple hematopoietic lineages is now well established as a powerful tool for studying hematopoietic

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differentiation and lineage restriction, and for generating unlimited numbers of hematopoietic stem and progenitor cell populations for transplantation [9–12]. ES or induced pluripotent stem (iPS) cells into hematopoietic cells have been differentiated by embryoid body (EB) formation or coculture with stromal cells, such as OP9 cells [13–16]. By using these protocols, several groups have previously established methods to generate mast cells from mouse [17–19], cynomolgus monkey [20], and human [21] ES cells. ES cell-derived mast cells could respond to stimulation with antigen and substance P by releasing histamine. However, in most cases, these cells do not develop the large granules and high levels of proteolytic enzymes characteristic of tissue mast cells.

In this study, we generated mast cells from mouse iPS cells (iPSMCs), and characterized them from the point of view of morphology, function, and gene expression. Our results showed that the iPSMCs that were differentiated by coculture with OP9 stromal cells or the EB formation method had characteristics similar to CTMCs. When iPSMCs that were generated on OP9 cells were cocultured with Swiss 3T3 fibroblasts, the iPSMCs exhibited a more functional phenotype.

Materials and Methods

Cell cultures

Two mouse iPS cell clones, 38C2 (a kind gift from Dr. S. Yamanaka, Kyoto University, Kyoto, Japan) [22] and 2A-EGFPTg-4F-01 [23], were used in the present study. These mouse iPS cells were routinely cultured in a leukemia inhibitory factor-containing ES cell medium (Specialty Media) on mytomycin C-treated mouse embryonic fibroblasts (MEFs; Specialty Media), and they were passaged every 2 days using 0.25% trypsin-EDTA (Invitrogen). OP9 stromal cells were cultured in an α-minimum essential medium (α-MEM: Sigma) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), and 1×nonessential amino acid (NEAA; Invitrogen).

Generation of BMMCs

C57BL/6 mice were purchased from Nippon SLC. Bone marrow cells were prepared from the femurs and tibiae of mice. Cells were cultured in an RPMI 1640 medium containing 10% FBS, 1×NEAA, and 10 ng/mL murine IL-3 (R&D Systems). The culture medium was replaced with a fresh medium every 5 days. After 4 weeks of culture, we confirmed the cellular surface expression of both FcεRI and c-kit (>95% positive).

Differentiation of iPS cells to mast cells

Before coculturing with OP9 cells or EB formation, mouse iPS cells were suspended in an ES cell medium and cultured on a culture dish at 37°C for 30 min to remove MEF layers. In the OP9 cell-mediated differentiation method, iPS cells were transferred onto OP9 cells in 6-well plates at a density of 1×10^4 cells per well. The induced cells were trypsinized on day 7, and 1×10^5 cells were seeded onto fresh OP9 cells with α-MEM supplemented with 20% FBS, 2 mM L-glutamine, 1×NEAA, 30 ng/mL IL-3, and 100 ng/mL SCF (Peprotech).

After 7 days, nonadherent cells were reseeded onto fresh OP9 cells. The cells were subcultured every 7 days. We harvested the differentiated cells on day 28 and used them for further analysis.

In the EB-mediated differentiation method, iPS cell-derived EBs were generated by culturing iPS cells on a round-bottom low-cell-binding 96-well plate at 1×10^3 cells per well. iPS cell-derived EBs were collected on day 7, and were transferred to a Petri dish with Differentiation Medium I [Dulbecco's modified Eagle's medium containing 15% FBS, 1×NEAA, 2 mM L-glutamine, 1×nucleosides, 0.1 mM 2-mercaptoethanol, penicillin/streptomycin, 30 ng/mL IL-3, 30 ng/mL IL-6 (Peprotech), and 100 ng/mL SCF]. After 7 days, nonadherent cells were transferred to a culture dish with Differentiation Medium II (Dulbecco's modified Eagle's medium containing 10% FBS, 1×NEAA, 2 mM L-glutamine, penicillin/streptomycin, 30 ng/mL IL-3, and 100 ng/mL SCF). We harvested the nonadherent cells on day 28 and used them for further analysis.

Transmission electron microscopy

BMMCs or iPSMCs were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), postfixed with 1% OsO_4, dehydrated by a graded ethanol series, passed through QY-1 (Nisshin EM), and then embedded in Epon-812 (TAAB,). Ultrathin sections (0.06-μm thick) were cut with an ultramicrotome (Leica Microsystems), stained with uranyl acetate–lead citrate, and observed using an electron microscope (H-7650, HITACHI) at 80 kV.

Protease assay

BMMCs or iPSMCs were washed with phosphate-buffered saline (PBS), lysed in PBS containing 2 M NaCl/0.5% Triton X-100, and incubated for 30 min on ice. The lysate was centrifuged at 12,000 rpm for 30 min at 4°C. Activities of granule proteases in the resultant supernatants were measured using their specific chromogenic peptide substrates, such as S-2288 for tryptase (Sekisui medical) and M-2245 for carboxypeptidase A (CPA; Bachem) [24].

### β-hexosaminidase release assay

β-hexosaminidase activity was measured as a marker of the granular fraction for evaluation of degranulation. Cells were washed with an HEPES buffer (137 mM NaCl, 20 mM HEPES, 5 mM D-glucose, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 2.4 mM $CaCl_2$, and 0.1% bovine serum albumin) and incubated with the buffer containing compound 48/80 (10 μg/mL; Sigma) or substance P (100 μM; Sigma) for 30 min. In the case of antigen stimulation, mast cells sensitized with 1 μg/mL anti-dinitrophenyl (DNP) IgE (SPE7; Sigma-Aldrich) for 24 h were stimulated with 100 ng/mL DNP-human serum albumin (HSA; Biosearch Technologies) in the presence of lysophosphatidylserine (Lyso-PS; Avanti Polar Lipids).

Coculture of mast cells with Swiss 3T3 fibroblasts

iPSMCs obtained after 28 days of culture with OP9 cells were cocultured with mitomycin C-treated Swiss 3T3 fibroblasts in the presence of 100 ng/mL SCF. BMMCs were

FIG. 1. Morphological characterization of induced pluripotent stem cell-derived mast cells (iPSMCs). (A) The iPSMCs, which were differentiated by coculture with OP9 cells or the embryoid body formation method, were harvested on day 28. Cytocentrifuged preparations of bone marrow-derived mast cells (BMMCs) and the iPSMCs were stained with May-Grunwald-Giemsa, toluidine blue, or Alcian blue and Safranin O solutions. (B) The ratio of Safranin O-positive cells to total cells was calculated and shown as a percentage. The data represent the means ± S.D. ($n = 4$). (C) BMMCs and iPSMCs were visualized by transmission electron microscopy. Scale bar = 2.0 μm.
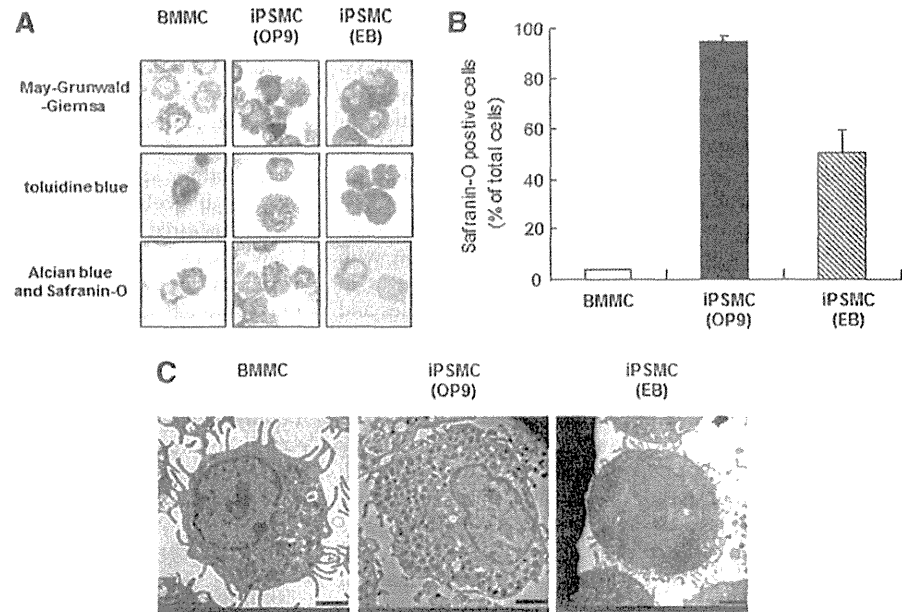
also cocultured with Swiss 3T3 fibroblasts under the same conditions. The subculture was performed every 4 days. The cells were trypsinized and replated, and nonadherent cells were collected as mast cells and used for further analysis.

Mast cell reconstitution and induction of passive cutaneous anaphylaxis

BMMCs or iPSMCs (5×10^5 cells) were injected subcutaneously into the conjunctivae of mast cell-deficient Kit$^{W-sh/W-sh}$
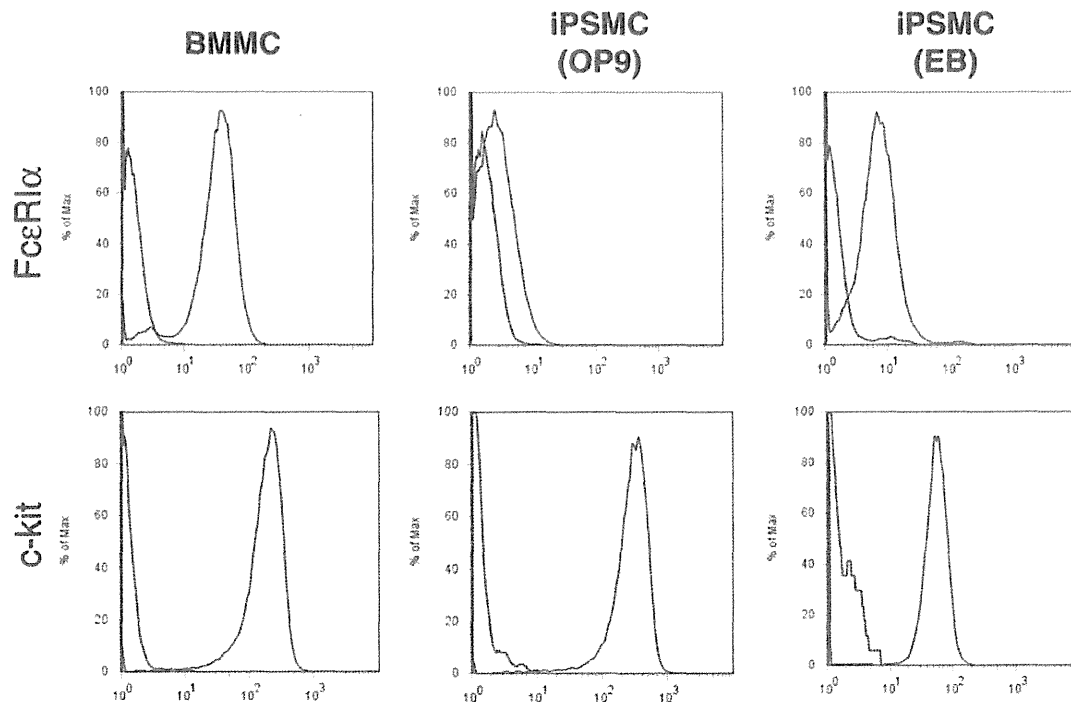

FIG. 2. Flow cytometric analysis of FcεRI and c-kit expression on iPSMCs. BMMCs and iPSMCs were stained with FITC-labeled anti-FcεRI and PE-labeled anti-c-kit antibodies for 30 min on ice. Stained cells were washed, resuspended in 1% fetal bovine serum–phosphate-buffered saline (FBS-PBS), and analyzed by flow cytometry.

mice. To elicit passive cutaneous anaphylaxis reactions, mice were injected subcutaneously into the conjunctiva with 75 ng anti-DNP IgE or saline. Then, 24 h after IgE injection, 100 μg DNP-HSA containing 2% Evan's blue dye was injected intravenously into mice. Thirty minutes later, the mice were killed, and their conjunctivae were excised. Evan's blue dye was extracted from conjunctivae with formamide, and the absorbance was measured at 610 nm.

Results

Generation of mast cells from mouse iPS cells

iPSMCs were generated by the OP9 coculture method or EB formation method as described in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub .com/scd). Approximately 6.5×10^6 mast cells could be obtained from 1×10^5 iPS cells by coculturing them with OP9 cells for 4 weeks. In addition, as in the case of BMMCs, iPSMCs can retain their proliferative potential (data not shown).

Next, we performed the staining with May-Grunwald-Giemsa, toluidine blue, Alcian blue, and Safranin O solutions. May-Grunwald-Giemsa staining of the iPSMCs, which were differentiated by coculture with OP9 stromal cells or the EB formation method (Supplementary Fig. S1), revealed that induced mast cells gave rise to a uniform phenotype with rough basophilic granule-containing cells (Fig. 1A, upper). The granules in these cells showed a metachromatic staining pattern when stained with acid toluidine blue (Fig. 1A, middle). We then performed Alcian blue and Safranin O staining, by which mast cells are known to show a specific red color if they are CTMCs and a blue color if they are immature mast cells or MMCs [1]. While BMMCs were Alcian blue positive and Safranin O negative, iPSMCs were positive for both Alcian blue and Safranin O staining (Fig. 1A, [lower], B). Electron microscopic analysis revealed that the iPSMCs differentiated by either method contained more granules than BMMCs (Fig. 1C).

Expression of high-affinity IgE receptor on iPSMCs

Mast cells are known to express c-kit and FcεRI (high-affinity IgE receptor) [1]. We next performed flow cytometric analysis to examine the surface expression of c-kit and FcεRI on iPSMCs. There was no significant difference in c-kit expression levels between iPSMCs and BMMCs (Fig. 2). In contrast, the FcεRIα expression level was significantly lower in the iPSMCs that were generated by coculture with OP9 cells, compared with that in BMMCs. Both c-kit$^+$FcεRI$^+$ and c-kit$^+$FcεRI$^-$ cells showed a granular phenotype by forward and side scatter (data not shown).

FcεRI is a heterotrimer composed of one α-chain and 2 γ-chains or a heterotetramer composed of one β-chain and 2 γ-chains. To evaluate the expression of each FcεRI subunit in iPSMCs, we analyzed mRNA expression levels by reverse transcription and quantitative polymerase chain reaction (RT-PCR). As shown in Supplementary Fig. S2, the expression levels of the mRNAs encoding the FcεRIα, FcεRIβ, and FcεRIγ chains were reduced in the iPSMCs differentiated by either method as compared with the levels in BMMCs.

Phenotypic differences between iPSMCs and BMMCs

To further compare the degree of mast cell differentiation, we measured the tryptase and CPA activities in iPSMCs. The tryptase and CPA activities were elevated in the iPSMCs derived from either method as compared with those in BMMCs (Fig. 3).

Histidine decarboxylase (HDC) is a critical enzyme that is involved in the synthesis of endogenous histamine in mammals [25–26], and is considered to be one of the indices of mast cell maturation [26]. Therefore, quantitative RT-PCR analysis was performed to compare the expression of HDC mRNA levels in iPSMCs and BMMCs (Supplementary Fig. S3). The expression level of HDC mRNA was elevated in the iPSMCs that were differentiated by either method as compared with that in BMMCs.
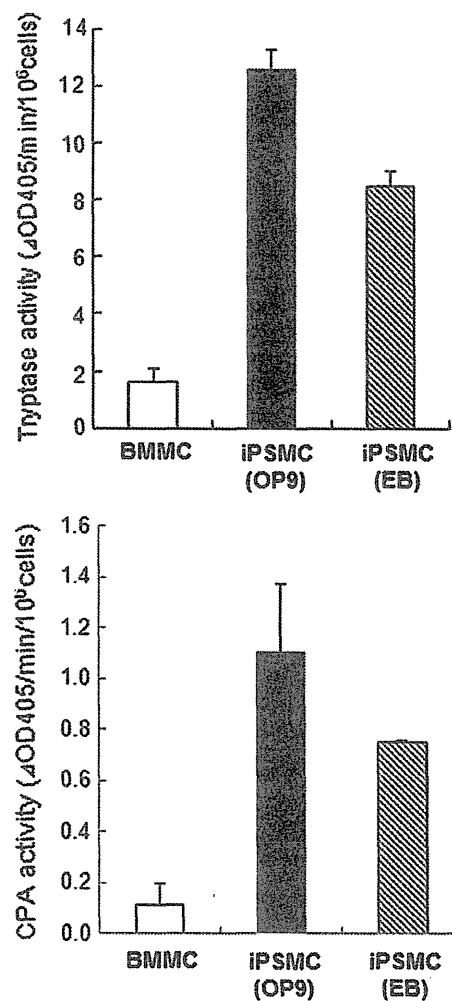

FIG. 3. Tryptase and carboxypeptidase A (CPA) activities in iPSMCs. Cell extracts prepared from BMMCs and iPSMCs were assayed for tryptase and CPA activities as described in the Materials and Methods section. All data represent the means ± S.D. ($n = 4$).
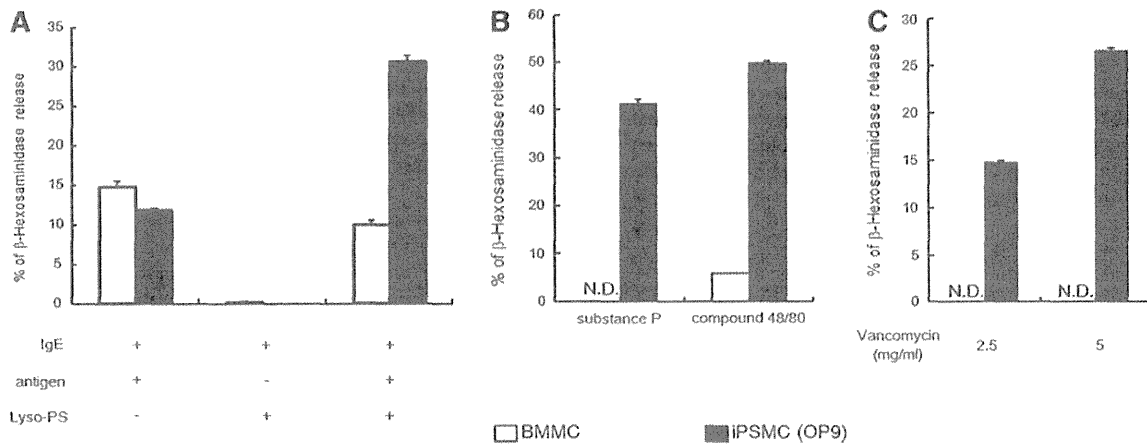
FIG. 4. β-hexosaminidase release from iPSMCs after stimulation with IgE/antigen, compound 48/80, substance P, and vancomycin. (A) The exocytotic response was determined by measuring the release of β-hexosaminidase. BMMCs (*open bar*) and the iPSMCs that were cocultured with OP9 cells (*closed bar*) were sensitized with anti-dinitrophenyl (DNP) IgE and stimulated with DNP human serum albumin (HSA) in the presence or absence of Lyso-PS as described in the Materials and Methods section. β-hexosaminidase enzymatic activity was measured in supernatants and cell pellets solubilized with 0.5% Triton X-100 in HEPES buffer. (B) BMMCs (*open bar*) and the iPSMCs that were cocultured with OP9 cells (*closed bar*) were stimulated with compound 48/80 or substance P. (C) BMMCs (*open bar*) and the iPSMCs that were cocultured with OP9 cells (*closed bar*) were stimulated with vancomycin. All data represent the means ± S.D. (*n* = 3).

Previously, Takano *et al.* demonstrated that *CD81*, a member of the tetraspanin superfamily, is one of the strikingly upregulated genes in BMMCs cocultured with Swiss 3T3 fibroblasts [8]. CD81 is also considered to be a marker of CTMCs. FACS analysis showed that expression of CD81 was elevated in the iPSMCs differentiated by either method as compared with that in BMMCs (Supplementary Fig. S4). In particular, our results revealed that the iPSMCs that were differentiated by coculture with OP9 cells were almost all CD81 positive and showed a homogeneous population.

Degranulation of iPSMCs

We compared FcεRI-mediated degranulation of BMMCs and iPSMCs by measuring the extracellular activity of β-hexosaminidase, a marker enzyme for histamine-containing granules. As shown in Fig. 4A, the iPSMCs that were differentiated by coculture with OP9 cells displayed significantly less release of β-hexosaminidase than the BMMCs in response to IgE-mediated antigen stimulation. Similar results were obtained in iPSMCs that were derived from EB formation methods (Supplementary Fig. S5A). On the other hand, when the iPSMCs that were pretreated with anti-DNP monoclonal IgE were incubated with DNP-HSA in the presence of Lyso-PS, which is known to enhance IgE-mediated degranulation in rat peritoneal mast cells [27], the amount of β-hexosaminidase release was increased.

Responses to cationic secretagogues such as compound 48/80 and substance P are functional characteristics of CTMCs [4]. We next compared the IgE-independent responses between BMMCs and iPSMCs. Stimulation of iPSMCs with compound 48/80 resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of this enzyme was detected from stimulated BMMCs (Fig. 4B and Supplementary Fig. S5B). Similarly, β-hexosaminidase release from

iPSMCs was much more markedly elevated by substance P treatment than β-hexosaminidase release from BMMCs (Fig. 4B and Supplementary Fig. S5B). In addition, stimulation of iPSMCs with vancomycin resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of β-hexosaminidase was detected from vancomycin-stimulated BMMCs (Fig. 4C and Supplementary Fig. S5C). These results indicate that the iPSMCs display a CTMC-like phenotype.

Comparison between iPSMCs differentiated by the OP9 coculture and EB formation protocols

We next compared the degree of differentiation between the iPSMCs that were differentiated by the OP9 coculture method and those differentiated by the EB formation method. The expression level of FcεRI was significantly lower in the iPSMCs that were differentiated by coculture with OP9 cells as compared with the iPSMCs that were differentiated by EB formation (Fig. 2). However, the number of Safranin O-positive cells was significantly greater in the iPSMCs that were cocultured with OP9 cells than in the iPSMCs that were derived from the EB formation method (Fig. 1B). In addition, the expression levels of HDC mRNA and CD81 protein were significantly higher in the iPSMCs that were cocultured with OP9 cells than in those that were derived from the EB formation method (Supplementary Figs. S3 and S4). These results showed that the iPSMCs that were cocultured with OP9 cells were more mature than the iPSMCs that were derived from the EB formation method. The iPSMCs that were derived from EB formation were more mature than BMMCs (Figs. 1–3). During the differentiation step, the iPSMCs that were derived from the EB formation method were designated as mast cells I (Fig. 5F). The iPSMCs that were differentiated by coculture with OP9 cells were also designated as mast cells II.
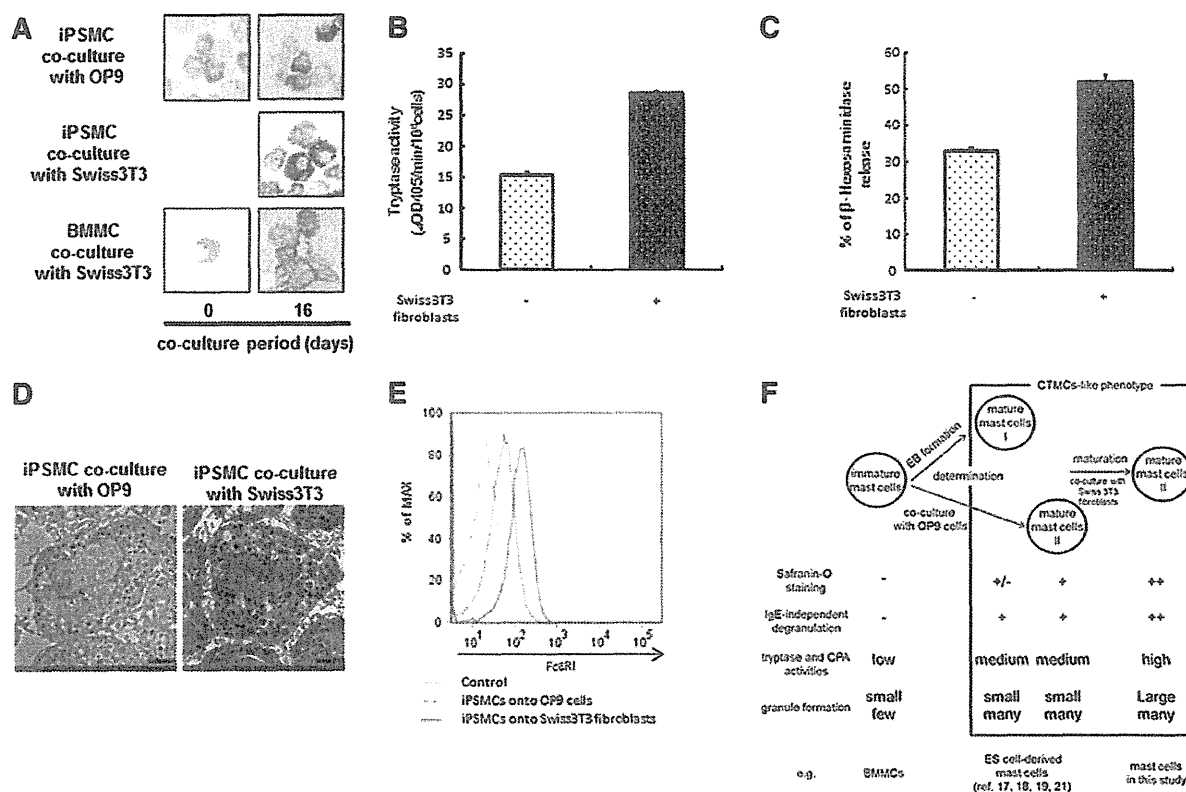
FIG. 5. Maturation of iPSMCs cocultured with Swiss 3T3 fibroblasts in the presence of stem cell factor. (A) Cytocentrifuged preparations of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts, and the BMMCs that were cocultured with Swiss 3T3 fibroblasts were stained with Alcian blue and Safranin O. (B) Granule protease activities of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were measured. (C) The exocytotic response was determined by measuring the release of β-hexosaminidase. The iPSMCs that were cocultured with OP9 cells (*open bar*) or Swiss 3T3 fibroblasts (*closed bar*) were stimulated with compound 48/80. (D) The iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were analyzed by transmission electron microscopy. Scale bar = 2.0 μm. (E) Suspensions of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were stained with FITC-labeled anti-FcεRI antibody for 45 min on ice. The stained cells were washed, resuspended in 1% FBS-PBS, and analyzed by flow cytometry. (F) Scheme of 2-step differentiation of mast cells from iPS cells. All data represent the means ± S.D. ($n = 3$).

Enhancement of maturation in iPSMCs by Swiss 3T3 fibroblasts

A previous study reported that coculture of BMMCs with Swiss 3T3 fibroblasts in the presence of SCF facilitated morphological and functional maturation toward a CTMC-like phenotype [8]. It is possible that Swiss 3T3 fibroblasts promote the maturation of the iPSMCs (mast cells II) that are generated on OP9 cells. Therefore, we expected to generate more mature iPSMCs by coculturing with Swiss 3T3 fibroblasts. We compared the degree of mast cell maturation of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts. Although the majority of BMMCs were Alcian blue positive and Safranin O negative, the percentage of Safranin O-positive mast cells was gradually increased up to ~80% on day 16 after coculturing with Swiss 3T3 fibroblasts (Fig. 5A). The staining intensity for Safranin O on the iPSMCs that were cocultured with Swiss 3T3 fibroblasts was stronger than the iPSMCs (mast cells II) (Fig. 5A). Therefore,

the iPSMCs that were differentiated by coculture with Swiss 3T3 fibroblasts were found to be more mature than mast cells II and designated as mast cells III (Fig. 5F). We measured the tryptase activity in the iPSMCs (mast cells II) or iPSMCs (mast cells III), and found the elevated tryptase activity in the iPSMCs (mast cells III) relative to the iPSMCs (mast cells II) (Fig. 5B). Similarly, β-hexosaminidase release by compound 48/80 in the iPSMCs (mast cells III) was markedly elevated in comparison with that in the iPSMCs (mast cells II) (Fig. 5C). Electron microscopic analysis of mast cells revealed that the iPSMCs (mast cells III) contained more large granules (Fig. 5D). We performed flow cytometric analysis to examine the surface expression of c-kit and FcεRI on the iPSMCs (mast cells III). After coculturing, iPSMCs (mast cells III) and BMMCs still expressed similar levels of c-kit (data not shown). Remarkably, the expression level of FcεRI was elevated in the iPSMCs (mast cells III) (Fig. 5E). These results showed that there were 2 steps in iPSMC maturation process.
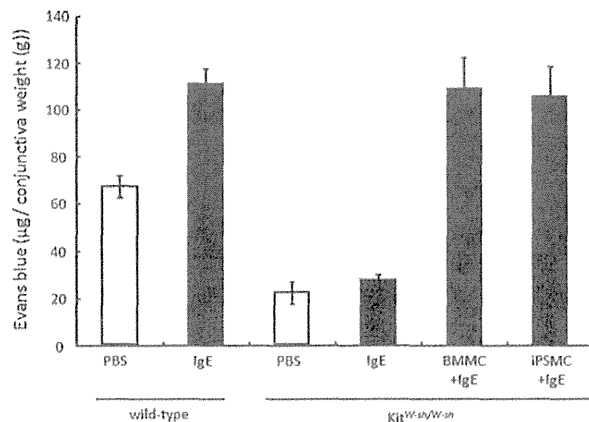
FIG. 6. IgE-dependent passive cutaneous anaphylaxis in mast cell-deficient Kit$^{W-sh/W-sh}$ mice reconstituted with iPSMCs. BMMCs or iPSMCs were injected subcutaneously into the conjunctivae of mast cell-deficient Kit$^{W-sh/W-sh}$ mice. After 6 weeks, mice were subcutaneously sensitized with anti-DNP IgE or saline, followed by induction of passive cutaneous anaphylaxis. After 24 h, mice were intravenously injected with DNP-HSA along with 2% Evan's blue dye. Thirty minutes later, conjunctivae were excised, and Evan's blue dye was extracted. Extravasation of Evan's blue dye was quantified as described in the Materials and Methods section. Results are normalized to average conjunctivae weight and are expressed as mean±S.D. (*n*=3 mice per group).

Mast cell reconstitution and induction of passive cutaneous anaphylaxis

We assessed whether the iPSMCs (mast cells I) with a C57BL/6 background could exhibit passive cutaneous anaphylaxis 6 weeks after injection of iPSMCs or BMMCs into the conjunctiva of mast-cell-deficient (Kit$^{W-sh/W-sh}$) mice [28]. Kit$^{W-sh/W-sh}$ mice reconstituted with BMMCs or iPSMCs exhibited passive cutaneous anaphylaxis reactions in the conjunctivae as measured by extravasation of Evan's Blue dye (Fig. 6). On the other hand, no passive cutaneous anaphylaxis reactions in the conjunctivae were observed for Kit$^{W-sh/W-sh}$ mice that were not reconstituted with mast cells. These results indicate that iPSMCs had the ability to respond to stimulation with IgE/antigen in vivo.

Discussion

In this study, we developed a protocol consisting of mesoderm induction (stage 1), mast cell specification (stage 2), determination of mast cells (stage 3), and maturation of mast cells (stage 4) for mast cell differentiation from iPS cells (Supplementary Figs. S1 and S6). BMMCs have been used extensively as a mast cell model. We compared the degree of maturation in BMMCs and iPSMCs. Expression level of CD81 was higher in the iPSMCs that were differentiated by both methods than in BMMCs (Supplementary Fig. S4). We also showed that IgE-mediated degranulation of iPSMCs was elevated in comparison with that in BMMCs in the presence of Lyso-PS (Fig. 4A and Supplementary Fig. S5A), demonstrating that iPSMCs were more mature than BMMCs. Therefore, both OP9 cells and EB-derived feeder cells might

moderately promote the maturation of mast cells. The other possibility is that the maturation of iPSMCs might be accelerated by SCF. SCF is one of the most important cytokines for mast cell maturation [29]. BMMCs are in general generated without SCF. These are reasons why iPSMCs were more mature than BMMCs with respect to their phenotypes and functions.

Cytokines and feeder cells were required to induce the mast cell development from mouse iPS cells. As previously described, IL-3 is known to play an important role in mast cell specification. In contrast, BMMCs are generated without OP9 cells, suggesting that OP9 cells are not necessary for mast cell specification. OP9 cells might promote the maturation of mast cells, possibly by OP9 cell-derived factors, such as IL-4 [30–31], IL-6 [32–34], and nerve growth factor [34]. Therefore, cytokines and OP9 cells are all-essential and have distinct roles in the differentiation of mast cells from iPS cells.

We found a difference in FcεRI expression levels between iPSMCs (mast cell I) and iPSMCs (mast cell II) (Fig. 2). Surface expression level of FcεRI was lower in the iPSMCs (mast cells II), compared with that in the iPSMCs (mast cells I). More recently, Kovarova et al. reported that expression of FcεRIα mRNA was lower in human ES cell-derived mast cells that were cocultured with OP9 cells than in human ES cell-derived mast cells that were derived from the EB formation method [21]. These findings were fully consistent with our results. However, our results showed that iPSMCs (mast cells II) were more mature than iPSMCs (mast cells I). These results indicate that the expression levels of FcεRI are not completely correlated with the degree of mast cell differentiation, although the iPSMCs (mast cells III) showed a high level of FcεRI expression (Fig. 5E).

In the present study, we demonstrated that, as in the case of BMMCs, Swiss 3T3 fibroblasts could promote the maturation of iPSMCs (Fig. 5). A recent study has reported that cynomolgus monkey ES cells that are cocultured with the murine aorta-gonad-mesonephros-derived stromal cell line AGM-S1 cells are differentiated into CTMCs [20]. These results suggest that feeder cells, including AGM-S1 and Swiss3T3 fibroblasts, would promote mast cell maturation by similar mechanisms. These feeder cells might contribute to the identification of factors that play a role in mast cell maturation.

While iPSMCs (mast cells II) were almost all Safranin O positive, iPSMCs (mast cells I) included both Safranin O-positive and negative populations, suggesting that immature cells were contained in iPSMCs (mast cells I). Expression levels of CD81 protein and HDC mRNA, and protease activities, were slightly elevated in the iPSMCs (mast cells II) as compared with the iPSMCs (mast cells I). Our results suggest that the iPSMCs (mast cells II) were more mature than iPSMCs (mast cell I). The iPSMCs (mast cells III) exhibited more mature phenotypes, such as large granules and high activity of protease. Taken together, the rank order of maturity in mast cells was the following: iPSMCs (mast cells III) > iPSMCs (mast cells II) > iPSMCs (mast cells I). The precise mechanisms of mast cell maturation process remain to be clarified. Classification of mast cells by using cell surface or internal marker can contribute to clarify the maturation mechanism of mast cells. Further studies are needed to find cell surface or internal marker that can clearly distinguish iPSMCs (mast cells I and II) and iPSMCs (mast cells III).

Our data demonstrate that iPSMCs could functionally respond to IgE stimulation in vivo (Fig. 6). There was no significant difference in Evan's blue extravasation in the $Kit^{W-sh/W-sh}$ mice reconstituted with iPSMCs or BMMCs. Previously, Fukuda et al. demonstrated that conjunctiva reconstituted with BMMCs display a CTMC-like phenotype [28]. Therefore, passive cutaneous anaphylaxis reactions were comparable in $Kit^{W-sh/W-sh}$ mice reconstituted with iPSMCs or BMMCs.

Galli's group first reported the identification of mast cell-committed progenitors (MCPs) in adult murine bone marrow [35]. They indicated that MCPs may be directly developed from multipotential progenitors independent of the myeloid pathway. In contrast, Arinobu et al. demonstrated that granulocyte/monocyte progenitors gave rise to MCPs [36]. The models of the developmental process in mast cells differed between these 2 reports. To analyze cells at each differentiation step, our differentiation protocol will be useful for clarifying the developmental process of mast cells.

Because of their pluripotency and self-renewal, ES cells and iPS cells are potential cell sources for regenerative medicine and other clinical applications, such as cell therapies, drug screening, toxicology, and investigation of disease mechanisms. Notably, iPS cell-based screening approaches might support the development of personalized medicine and tailor-made treatment plans. Vancomycin, an antibiotic to which methicillin-resistant *Staphylococcus aureus* (MRSA) is sensitive, frequently induces allergic reaction [37]. In this study, the stimulation of the iPSMCs with vancomycin resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of this enzyme was detected from BMMCs (Fig. 4C and Supplementary Fig. S5C). Therefore, iPSMCs would be potential cell sources for drug-allergy-screening system.

We developed a 2-step differentiation protocol of mast cells from iPS cells. In the conventional method, CTMC-like mast cells are produced from bone marrow cells after 45 days of culture. In contrast, the iPSMCs generated on OP9 cells in the present study were produced after 28 days of culture. Thus, homogeneous CTMC-like mast cells can be easily generated from iPS cells by the OP9 coculture method. On the other hand, Swiss 3T3 coculture methods have different advantage from OP9 coculture systems. The iPSMCs that were cocultured with Swiss 3T3 fibroblasts were more mature than the iPSMCs that were generated on OP9 cells. Because each of these methods has its advantages, the protocol should be chosen in accordance with the intended use.

We successfully developed a 2-step differentiation protocol for generating more mature mast cells from mouse iPS cells. The iPSMCs generated in this study exhibit many characteristics distinct from BMMCs. The iPSMCs possessed the characteristics of mature mast cells, including the heparin contents and degranulation, in response to cationic secretagogues and vancomycin. The iPSMCs serve as an excellent model for in vitro studies of CTMCs. Our results could facilitate clarification of the mechanisms that control the development of mast cells.

Acknowledgments

Author Disclosure Statement

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Type-I IFN signaling is required for the induction of antigen-specific CD8$^+$ T cell responses by adenovirus vector vaccine in the gut-mucosa

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ABSTRACT

Adenovirus vector (Adv) vaccination at a systemic site, such as intramuscular (i.m.) immunization, can induce antigen-specific CD8$^+$ T cell responses in both systemic and mucosal compartments. It remains unclear, however, how antigen-specific CD8$^+$ T cell response is induced in the mucosa. In this study, we found that type-I IFN signaling is required for the induction of mRNA expression of retinal dehydrogenase in the draining lymph nodes following the i.m. Adv vaccination. We show that type-I IFN signaling is required for the induction of antigen-specific CD8$^+$ T cell response in the gut-mucosal compartment following the i.m. Adv vaccination.

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1. Introduction

Replication-incompetent recombinant adenovirus vectors (Adv) that can efficiently transduce exogenous genes are broadly used as gene delivery tools. Because antigen delivery by Adv can induce potent antigen-specific cellular and humoral immune responses in the systemic compartment [1], it is anticipated that Adv vaccine will be effective against infectious pathogens, such as human immunodeficiency virus and influenza virus [2,3]. It was recently reported that the intramuscular (i.m.) immunization of the Adv-expressing simian immunodeficiency virus (SIV)-*gag* induced sustainable and functional SIV-*gag*-specific CD8$^+$ T cell responses in mucosal as well as systemic compartments in mice and rhesus macaques [4–6]. In addition, Ganguly et al. and Kaufman et al. showed that the i.m. Adv vaccine promotes the production of retinoic acid (RA) in dendritic cells (DCs) of draining lymph nodes (DLNs) [6,7]. RA, a vitamin A metabolite, is produced by aldehyde dehydrogenase (Aldh) 1a (retinal dehydrogenase (RALDH)) and is required

for imprinting gut-homing capacity on T and B cells [8,9]. Ganguly et al. showed that the RA-dependent upregulation of $\alpha_4\beta_7$ integrin, a gut-homing molecule, on CD8$^+$ T cells induced by Adv requires the activation of nuclear factor-κB (NF-κB) in conventional DCs (cDCs) *in vitro* [6].

Recently, it has been clearly indicated that the innate immune response is essential for effective induction of the adaptive immune response. It is increasingly being considered that the strong induction of antigen-specific CD8$^+$ T cells by Adv vaccine is due to innate immune responses against Adv. In fact, Rhee et al. and others reported that the induction of antigen-specific CD8$^+$ T cell responses by Adv vaccine is significantly reduced in both systemic and mucosal compartments of mice lacking myeloid differentiation protein-88 (MyD88) [10–13]. These results indicate that innate immune signaling is required for the induction of antigen-specific CD8$^+$ T cell responses by Adv vaccine in both systemic and mucosal compartments. However, the antigen-specific immune response was not completely diminished in MyD88-deficient mice [10–13], suggesting that other innate immune signalings are involved in the Adv-mediated antigen-specific CD8$^+$ T cell response in the systemic and mucosal compartments.

The induction of antiviral innate immune response begins with the recognition of viral components by host pattern recognition

receptors (PRRs). The pathogen-recognition by these receptors promotes the maturation of antigen-presenting cells (APCs), and then the activated APCs produce various cytokines which are required to trigger the pathogen-specific adaptive immune response [14–16]. We and others have reported that Adv induces the production of inflammatory cytokines such as IL-6 and IL-12 in cDCs by Toll-like receptor (TLR) 9/MyD88-dependent signaling [17,18]. Zhu et al. showed that the production of type-I IFN by Adv in cDCs is independent of the TLR9/MyD88 signaling, whereas it is dependent on TLR9/MyD88 signaling in plasmacytoid DCs (pDCs) [19]. These observations indicate that Adv can induce innate immune responses through TLR9/MyD88-dependent and – independent signaling. The retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), including RIG-I and melanoma differentiation-associated gene 5 (Mda5), both of which are expressed in most cell types, are known to recognize cytosolic RNAs [20–22]. RIG-I and Mda5 transduce intracellular signaling through IFN-β promoter stimulator-1 (IPS-1) [23]. This signaling then leads to the production of inflammatory cytokines and type-I IFN. Perreau et al. showed that the levels of type-I IFN positively correlated with titers of Ad5-specific NAbs, suggesting a possibility that type-I IFN signaling controls in the efficacy of Adv vaccine [24]. Thus, it is important to elucidate the effect for adaptive immune responses through the activation of type-I IFN signaling induced by Adv vaccination. We previously reported that IPS-1 is involved in the production of IFN-β in mouse embryonic fibroblasts (MEFs) stimulated by Adv, and Adv-derived virus-associated RNAs (VA-RNAs) which are transcribed by RNA polymerase III are key factors in this process [25]. However, it is unclear whether IPS-1 signaling is involved in the immunogenicity of Adv vaccine.

In this study, we found that IPS-1 and type-I IFN signaling promotes the expression of IFN-β, GM-CSF and RA in the DLNs following the i.m. Adv vaccine. In addition, we found that IPS-1 and type-I IFN signaling are required for the induction of antigen-specific CD8+ T cell responses following i.m. Adv vaccination in the gut-mucosal compartment but not the systemic compartment. These results suggest that type-I IFN signaling is required for the induction of antigen-specific CD8+ T cell responses in the gut-mucosal compartment in an RA-dependent manner following the i.m. Adv vaccine.

2. Materials and methods

2.1. Mice

C57BL/6J (wild-type; WT) mice were purchased from Japan SLC (Hamamatsu, Japan), and IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice (C57BL/6J background) were established as described previously [26]. All mice were housed in an animal facility under a specific-pathogen-free condition and were used at 6–8 weeks of age. All animal experimental procedures used in this study were performed in accordance with the institutional guidelines for animal experiments at Osaka University and the National Institute of Biomedical Innovation.

2.2. Adv production and immunization

The adenovirus type 5 vector-expressing LacZ (Ad-LacZ) was constructed as described previously [27]. Briefly, the expression cassette containing the β-actin promoter with the cytomegalovirus enhancer (CA)-driven [28] LacZ gene was inserted into the E1/E3-deleted adenovirus type 5 genome. This virus was grown in 293 cells by standard techniques. Ad-LacZ was purified with CsCl$_2$ step-gradient ultra-centrifugation, dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM MgCl$_2$, and 10% glycerol, and stored in aliquots at −80 °C. Determination of the virus particle (vp) titers was accomplished spectrophotometrically according to the methods of Maizel et al. [29].

All mice were injected under anesthesia in the right and left quadriceps muscles with Ad-LacZ (5×10^9 vp per muscle; total 10^{10} vp per mouse).

2.3. Isolation of mononuclear cells

Peripheral blood mononuclear cells were obtained after lysing red blood cells. Splenocytes were isolated by a standard mechanical disruption procedure, followed by lysis of the red blood cells, and then both components were passed through a nylon mesh. Small bowel lamina propria (LP) lymphocytes were isolated by a standard enzymatic dissociation procedure [30]. In brief, small bowel specimens were removed from Peyer's patches and then cut into small pieces using scissors. These pieces were washed with phosphate-buffered saline (PBS) and then stirred in RPMI 1640 containing 10% fetal calf serum (FCS) and 2 mM EDTA at 37 °C for 20 min to remove intraepithelial cells. The pieces were vigorously shaken, washed five times with PBS to remove EDTA, and then minced and digested using RPMI 1640 containing 10% FCS, collagenase type D (Roche, GmbH, Germany) and DNase I (Roche) at 37 °C for 30 min. Mononuclear cells were then isolated by a discontinuous density gradient procedure (40% and 75%) with Percoll PLUS (GE Helthcare, Tokyo, Japan). The cells that were layered between the 40% and 75% interface were collected as small bowel LP lymphocytes.

2.4. Reverse transcription and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from inguinal lymph nodes (iLN) lysates using an RNeasy Mini Kit (Qiagen, GmbH, Germany). Total RNA (500 ng) was used to synthesize cDNA using SuperScript VILO (Invitrogen, Carisbad, CA, USA) according to the manufacturer's instructions. The synthesized cDNA was used as a template for real-time PCR, which was performed in triplicate with SYBR Premix Ex Taq™ II (TaKaRa, Shiga, Japan) and each gene-specific primer (*Ifna* forward, 5′-CTTCCACAGGATCACTGTGTACCT-3′; *Ifna* reverse, 5′-TTCTGCTCTGACCACCTCCC-3′; *Ifnb* forward, 5′-CTGGAGCAGCTGAATGGAAAG-3′; *Ifnb* reverse, 5′-CTTCTCCGTCATCTCCATAGGG-3′; *Csf2* forward, 5′-TTTTCCTGGGCATTGTGGTC-3′; *Csf2* reverse, 5′-GGCATGTCATCCAGGAGGTT-3′; *Aldh1a1* forward, 5′-ATGGTTTAG-CAGCAGGACTCTTC-3′; *Aldh1a1* reverse, 5′-CCAGACATCTTGAATC-CACCGAA-3′; *Aldh1a2* forward, 5′-GACTTGTAGCAGCTGTCTTCACT-3′; *Aldh1a2* reverse, 5′-TCACCCATTTCTCTCCCATTTCC-3′; *Aldh1a3* forward, 5′-GGACAGTCTGGATCAACTGCTAC-3′; *Aldh1a3* reverse, 5′-TCAGGGGTTCTTCTCCTCGAGT-3′; *Gapdh* forward, 5′-CAATG TGTCCGTCGTGGATCT-3′; *Gapdh* reverse, 5′-GTCCTCAGTGTAG CCCAAGATG-3′). PCR and analysis were performed on an Applied Biosystems StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative expression was calculated using the ΔΔCT method, and the mRNA level of each gene was normalized with that of *Gapdh*.

2.5. Tetramer-binding assay

The tetramer-binding assay was performed as previously described [31]. In brief, 1×10^6 lymphocytes from blood, spleen or small bowel LP were incubated with anti-CD16/32 antibody (Ab) (Fc block; eBioscience, San Diego, USA) for 15 min at 4 °C, stained with phycoerythrin (PE)-conjugated H-2K^b/β-gal$_{96-103}$ (DAPIYTNV) tetramer reagent (MBL, Nagoya, Japan), and then stained with allophycocyanin (APC)/Cy7-conjugated anti-mouse CD3ε Ab (145-2C11, BioLegend, San Diego, USA) and eFlour™ 450-conjugated anti-mouse CD8α Ab (Ly-2, eBioscience). The stained cells were

analyzed by an LSR II flow cytometer and BD FACSDiva™ Ver 6.1 software (BD Bioscience). Dead cells were excluded by 7-amino-actinomycin D staining (eBioscience).

2.6. Statistics

All results are shown as the mean ± standard error of the mean. Statistical significance was analyzed by the one-way ANOVA among groups. $^*p < 0.05$, $^{**}p < 0.01$; compared to WT mice at 8 h. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$; compared to the expression levels of each group at 0 h.

3. Results

3.1. Type-I IFN signaling promotes innate immune responses and the production of RA in draining lymph nodes following i.m. Adv vaccination

It has been clearly shown that the activation of innate immune responses is essential for the effective induction of an adaptive immune response. We previously reported that IPS-1 signaling activated by Adv induces the production of type-I IFNs in MEFs [25]. In addition, it was reported that the iLNs are anatomic sites for the priming and early trafficking of vaccine-induced CD8$^+$ T cells following i.m. administration of Adv vaccine [7]. We speculated that the Adv vaccine imprints the capacity of these CD8$^+$ T cells to migrate into gut-mucosa in iLN through the production of type-I IFN.

To examine this hypothesis, we first analyzed the mRNA expression of Ifna, Ifnb and Csf2 (GM-CSF) in the iLN of WT mice, IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice by RT-qPCR at 8 h after the i.m. administration of Ad-LacZ when the highest expression of Aldh1a1 in the iLN is induced as shown in a previous report [7]. As the result, we found that the i.m. Ad-LacZ elicited the upregulation of Ifna mRNA in WT mice, IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice (Fig. 1A).

The i.m. Ad-LacZ also elicited the upregulation of Ifnb and Csf2 in WT mice, and to a lesser degree in IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice (Fig. 1B and C). It has been reported that RA, a vitamin A metabolite, imprints the gut-homing capacity on T and B cells, and the RA production in DCs was induced by GM-CSF [32] or stimulation through TLR [33]. In addition, DCs express Aldh1a2 and stromal cells barely express Aldh1a1 in LNs [34]. Thus, to investigate whether type-I IFN signaling is involved in the promotion of RA production in the iLN, we also analyzed the mRNA expression of Aldh1a1, Aldh1a2 and Aldh1a3 in the iLN of WT mice, IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice. We observed the upregulation of Aldh1a1 but not Aldh1a2 and Aldh1a3 in WT mice by the immunization with Ad-LacZ. On the other hand, Aldh1a1 was not upregulated, and Aldh1a2 and Aldh1a3 were severely downregulated by the i.m. Ad-LacZ in IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice (Fig. 1D–F). Taken together, these findings suggest that type-I IFN signaling promotes the production of GM-CSF and RA in the iLN following i.m. Adv vaccination.

3.2. Type-I IFN signaling is required for the induction of antigen-specific CD8$^+$ T cell responses by Adv vaccine in the gut-mucosal compartment

Next, we speculated that the downregulation of Aldh1a expression might cause the reduction of RA production in IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice, and that the reduced RA production would not sufficiently induce antigen-specific CD8$^+$ T cells in the gut-mucosal compartment following Adv vaccination. To examine this hypothesis, we measured the frequencies of antigen-specific CD8$^+$ T cells in the blood, spleen and small bowel LP of the immunized WT mice, IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice. Interestingly, the frequencies of β-gal-specific (H-2K^b/β-gal$_{96-103}$ (DAPIYTNV) tetramer$^+$) CD8$^+$ T cells in the small bowel LP from IPS$^{-/-}$ mice and IFNAR2$^{-/-}$ mice immunized with Ad-LacZ were significantly reduced as compared with that in WT mice immunized with Ad-LacZ, although the frequencies were equivalent in the blood and
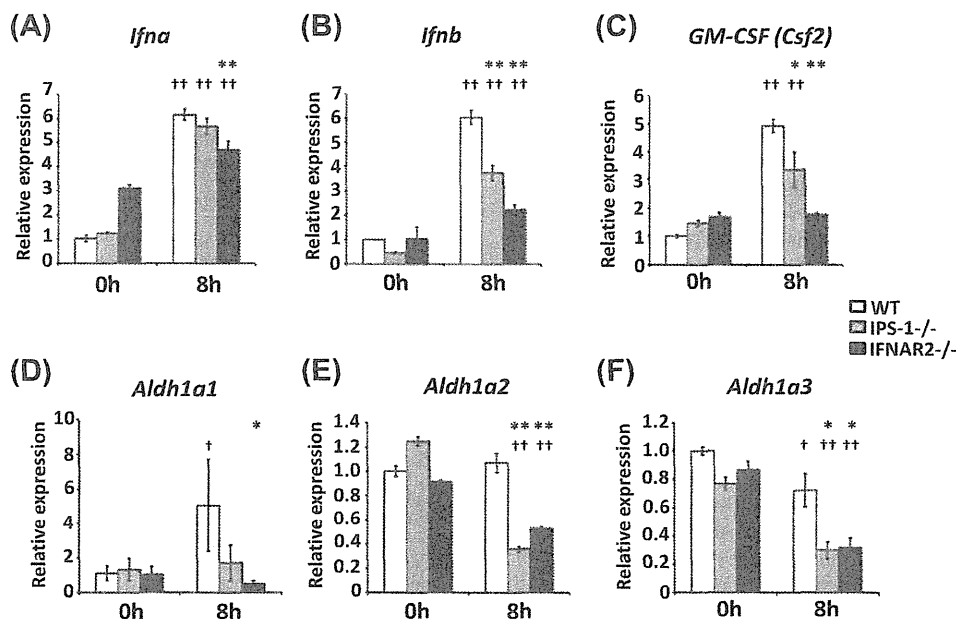

Fig. 1. Relative expression of type-I IFN, GM-CSF and ALDH mRNA in iLN from IPS-1$^{-/-}$ and IFNAR2$^{-/-}$ mice i.m. immunized with Ad-LacZ. At 8 h after the i.m. immunization of 10^{10} vp of Ad-LacZ, total RNA was extracted from whole iLN in WT mice, IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice. cDNA was synthesized from total RNA, and then expressions of type-I IFN (Ifna (A) and Ifnb (B)), GM-CSF (Csf2) (C) and ALDH1A enzymes (Aldh1a1 (D), Aldh1a2 (E) and Aldh1a3 (F)) mRNA were measured by RT-qPCR, normalized by GAPDH mRNA. The graphs represent the relative expression of each gene normalized by that of WT mice at the hour 0. Data are shown as the means ± SEM (n = 3) and are representative of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$.
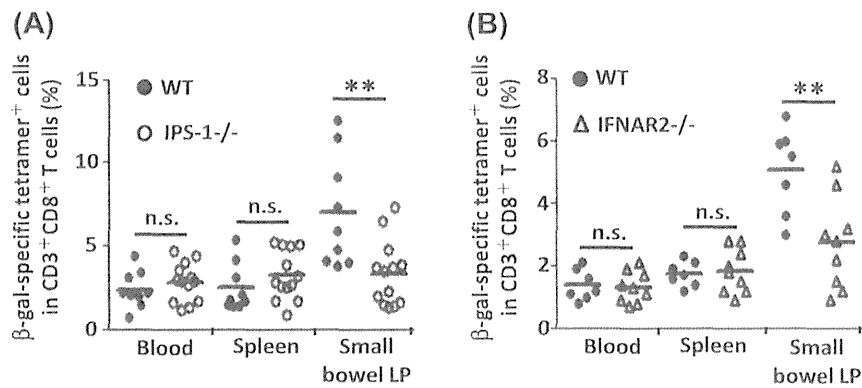
Fig. 2. The frequency of antigen-specific CD3⁺CD8⁺ T cells induced in systemic and gut-mucosal compartments of WT mice, IPS-1$^{-/-}$ mice and IFNAR2$^{-/-}$ mice by the i.m. immunization with Ad-LacZ. At 2 weeks after the i.m. 10^{10} vp of Ad-LacZ immunization, the frequency of β-gal-specific CD3⁺CD8⁺ T cells was measured by H-2K^b/β-gal$_{96-103}$ tetramer in the blood, spleen (systemic) and small bowel LP (gut-mucosal) from C57BL/6J wild-type (WT) mice ($n = 9$), and IPS-1$^{-/-}$ mice ($n = 13$) (A), WT mice ($n = 7$) and IFNAR2$^{-/-}$ mice ($n = 9$) (B). Bars indicate averages, and closed circles (WT), open circles (IPS-1$^{-/-}$) or open triangles (IFNAR2$^{-/-}$) indicate individual mouse samples. n.s. indicates not significant. Data pools are representative of three independent experiments. $^{**}p < 0.01$.

spleen among the strains (Fig. 2A and B). Therefore, these results indicate that type-I IFN signaling is required for the induction of antigen-specific CD8⁺ T cell responses to Adv vaccine in the gut-mucosal compartment but not in the systemic compartment.

4. Discussion

In this study, we showed that type-I IFN signaling activated by the i.m. Adv vaccine promotes the mRNA expression of *Ifnb, Csf2* and *Aldh1a1* and is required for the induction of antigen-specific CD8⁺ T cells by Adv vaccine in the gut-mucosal compartment but not the systemic compartment. We observed that the expression of IFN-β, but not IFN-α, after the i.m. immunization of Adv vaccine was prominently affected by IPS-1 or IFNAR2 deficiency. Since the production of IFN-α induced by Adv infection in MEF was undetectable (data not shown), it is speculated that IFN-α would be mainly produced by other type of cells, including DC, and that IFN-β would be produced by fibroblasts such as stromal cells by the i.m. immunization of Adv vaccine. We previously reported that the production of IFN-β in MEF induced by Adv is dependent on IPS-1 signaling and that in DC is partially dependent on IPS-1 signaling [25]. In addition, since previous reports have shown that type-I IFN amplifies its expression through IFNAR [35,36], it is suggested that the expression of IFN-β was severely decreased in IFNAR2$^{-/-}$ mice. Therefore, it is speculated that IPS-1 and type-I IFN signaling contribute to IFN-β, but not IFN-α, production from stromal cells in iLN following the i.m. immunization of Adv vaccine.

It was shown that the stromal cells barely express *Aldh1a1* in LNs [34] and the expression of *Aldh1a1* but not *Aldh1a2* in the iLN of C57BL/6 mice was upregulated following the i.m. immunization of Adv vaccine [7]. From these observations, it is speculated that *Aldh1a1* in the stromal cells of iLN would be induced by Adv vaccination. In support of this hypothesis, we observed the upregulation of *Aldh1a1* in WT mice by the immunization with Adv vaccine. We also observed that the expression of *Aldh1a2* and *Aldh1a3* in the iLN of Adv-immunized WT mice did not change, as was similarly shown for *Aldh1a2* expression in a previous report [7]. On the other hand, Ganguly et al. showed that the i.m. Adv vaccine upregulated the expression of *Aldh1a2* and *Aldh1a3* in cDCs of DLNs [6]. In our study, the expression of *Aldh1a2* and *Aldh1a3* was measured in the whole iLNs consisting of the T and B cells which do not express *Aldh1a2* and *Aldh1a3* and a few cDCs following the i.m. immunization of Adv vaccine. Therefore, we could observe a

relatively decreased mRNA expression in cDCs. Taken together, it is suggested that at least the *Aldh1a1* expression contributes to the imprinting of the capacity of antigen-specific CD8+ T cells to migrate into the gut-mucosa.

As shown here, we propose the importance of type-I IFN signaling for the induction of antigen-specific CD8⁺ T cell responses in the gut-mucosal compartment by Adv vaccine. It is hoped that these findings will contribute to the development of novel vaccines and adjuvants that can enhance the induction of mucosal immune response by immunization through systemic routes.

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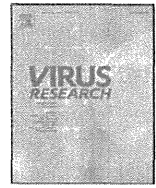
Improving adenovirus vector-mediated RNAi efficiency by lacking the expression of virus-associated RNAs[☆]

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ARTICLE INFO

ABSTRACT

Several studies have reported that short hairpin RNA (shRNA)-mediated RNA interference (RNAi) was competitively inhibited by the expression of adenovirus (Ad)-encoded small RNAs (VA-RNAs), which are expressed from a replication-incompetent Ad vector, as well as a wild-type Ad; however, it remained to be clarified whether an shRNA-expressing Ad vector-mediated knockdown was inhibited by VA-RNAs transcribed from the same Ad vector genome. In this study, we demonstrated that a lack of VA-RNA expression from the Ad vector leads to an increase in knockdown efficiencies of Ad vector-mediated RNAi. In the cells transduced with a first-generation Ad vector (FG-Ad) expressing shRNA (FG-Ad-shRNA), the copy numbers of shRNA and VA-RNAs incorporated into the RNA-induced silencing complex (RISC) was comparable. In contrast, higher amounts of shRNA were found in the RISC when the cells were transduced with an shRNA-expressing helper-dependent Ad (HD-Ad) vector, in which all viral genes, including VA-RNAs, were deleted (HD-Ad-shRNA), compared with FG-Ad-shRNA. HD-Ad vectors expressing shRNA against luciferase and p53 showed 7.4% and 37.3% increases in the knockdown efficiencies compared to the corresponding FG-Ad-shRNA, respectively, following in vitro transduction. Furthermore, higher levels of knockdown efficiencies were also found by the transduction with shRNA-expressing Ad vectors lacking VA-RNA expression (AdΔVR-shRNA) than by transduction with FG-Ad-shRNA. These results indicate that VA-RNAs expressed from an Ad vector inhibit knockdown by the shRNA-expressing Ad vector and that HD-Ad-shRNA and AdΔVR-shRNA are a powerful framework for shRNA-mediated knockdown.

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1. Introduction

RNA interference (RNAi) has become one of the most important techniques for both gene-function analysis in basic research and silencing of disease-related genes in clinical applications. In addition to chemically synthesized small interfering RNA (siRNA), vectors expressing short hairpin RNA (shRNA) under the control of RNA polymerase III promoters and RNA polymerase II promoters are widely used to induce RNAi in mammalian cells (Giering et al., 2008; Scherer and Rossi, 2003; Tuschl, 2002). The shRNA-based systems for RNAi have various advantages. First, shRNA expression can be controlled by various types of promoters. Not only the

ubiquitous U6 and H1 promoters but also tissue-specific promoters, including the liver-specific apolipoprotein E enhancer/human α1-antitrypsin promoter, are available for shRNA-mediated knockdown (Giering et al., 2008). Second, an shRNA expression cassette is efficiently delivered to targeted cells not only by non-viral vectors, but also by viral vectors, such as adenovirus (Ad), lentivirus, and adeno-associated virus vectors. An shRNA-expressing vector can be selected, depending on the situation, since each shRNA-expressing vector has distinct gene-transfer properties and activities (Grimm and Kay, 2007).

Among the various types of vectors for shRNA expression, Ad vectors are highly promising as potential shRNA-expressing vectors because of their superior transduction properties (Benihoud et al., 1999; Koizumi et al., 2007; Xu et al., 2005). We and other groups previously demonstrated that Ad vector-mediated RNAi has great potential for use as an shRNA-expressing vector in a variety of applications (Hosono et al., 2005; Hosono et al., 2004; Mizuguchi et al., 2007; Motegi et al., 2011). However, it was reported that virus-associated RNA (VA-RNA) I (a major species) and VA-RNA II (a minor species), which are small RNAs transcribed from the

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