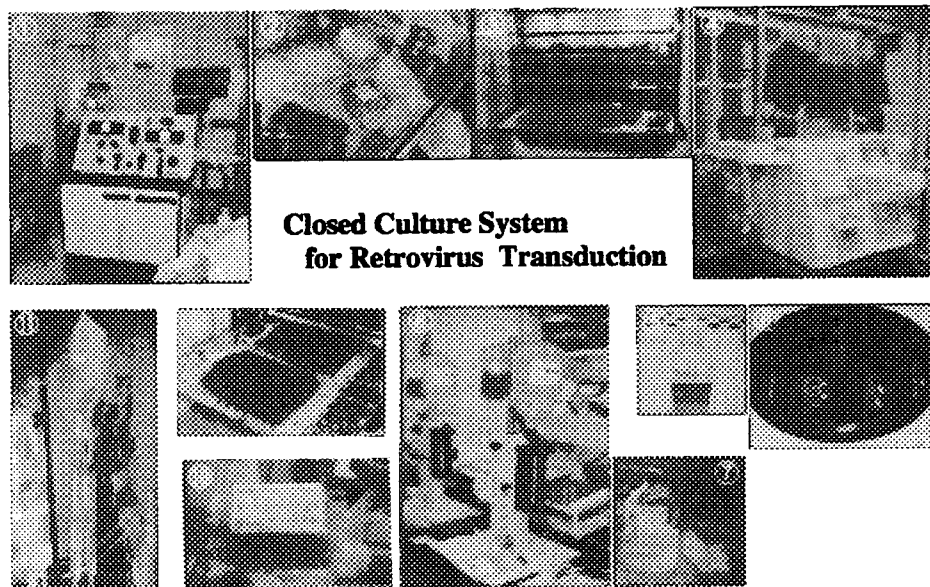


**Table 2.** Patients enrolled in Tsukuba TK-DLI Trial

UPN	Diagnosis	Age, Sex	# of prep (/kg)	NGFR+	# of infused (/kg)	GvHD
1	MDS (RAEB)	42, M	1.0x10 <sup>10</sup> (1.8x10 <sup>8</sup> )	93.5%		
2	ALL (Ph1+)	15, F	4.6x10 <sup>9</sup> (1.2x10 <sup>8</sup> )	97.8%		
3	AML	60, M	1.0x10 <sup>10</sup> (2.3x10 <sup>8</sup> )	97.2%	3.8x10 <sup>9</sup> (7.7x10 <sup>7</sup> )	acute (grade III)
4 <sup>1</sup>	ALL	20, M	0.1x10 <sup>9</sup> (0.2x10 <sup>7</sup> )	37.7%		
5 <sup>1</sup>	ALL		4.4x10 <sup>9</sup> (8.8x10 <sup>7</sup> )	93.1%		
6	MDS (RAEB)	58, M	2.4x10 <sup>10</sup> (3.1x10 <sup>8</sup> )	95.1%	9.7x10 <sup>9</sup> (9.5x10 <sup>7</sup> )	chronic
7-1	ALL	14, M	7.9x10 <sup>9</sup> (2.6x10 <sup>8</sup> )	94.9%	2.0x10 <sup>9</sup> (6.7x10 <sup>7</sup> )	
-2					5.0x10 <sup>9</sup> (8.5x10 <sup>7</sup> )	
8-1	AML	46, M	1.8x10 <sup>10</sup> (2.3x10 <sup>8</sup> )	90.7%	9.0x10 <sup>9</sup> (8.5x10 <sup>7</sup> )	
-2					9.0x10 <sup>9</sup> (8.5x10 <sup>7</sup> )	
9-1	ALL	50, M	7.2x10 <sup>9</sup> (1.8x10 <sup>8</sup> )	90.7%	4.4x10 <sup>9</sup> (8.6x10 <sup>7</sup> )	
-2					2.1x10 <sup>9</sup> (4.1x10 <sup>7</sup> )	

<sup>1</sup>an identical patient



**Figure 3.** The closed culture system for transduction into peripheral lymphocytes. Collection of donor lymphocytes using CS3000plus (1), cell culture in gas-permeable culture bags (2, 3), cell washing using Cytamate (4), SFCMM-3 virus sup (5), transduction by spinoculation (6), anti-NGFR antibody (7), isolation of transduced cells using Isoplex 50 (8), culture and collection of transduced cells (9, 10), and infusion into patients (11).

transduction efficiency was approximately 20% and the purity of NGFR-expressing cells isolated using Isoplex 50 exceeded 90% in all the cases except for UPN 4. All samples cleared the safety tests including cell viability, sterility, existence of replication competent retrovirus (RCR), and sensitivity to GCV. Five out of 8 patients (2 AML, 2 ALL, and 1 MDS) were treated with TK-DLI and three (UPN 7, 8, and 9) among them received the infusions twice (Table 2). The number of cells infused, although it varied among patients, was approximately  $8.7 \times 10^7$  per kilogram of body weight. Four patients showed some clinical responses such as inhibition of leukemic cell proliferation, mitigation of lymph node swelling, and lowering the values of tumor markers. Especially, a MDS patient (UPN 6) achieved complete remission and has remained in CR for 2 years after the treatment. Regarding GvHD occurrence, a patient (UPN 3) developed the grade III GvHD due to severe liver dysfunction that was successfully controlled by administration of GCV without any immunosuppressive drugs. Another patient (UPN 6)

showed chronic GvHD with precordial erythema that has been observed without any treatments. No adverse effects related to gene therapy have been observed.

## 5. PROBLEMS IN TK-DLI

Although TK-DLI proved to be clinically beneficial, it also has several critical problems. One of the major limitations of TK-DLI is considered to be the strong immunogenicity of the viral protein, HSV-TK. Two reports revealed that infusions of the transduced cells into immune-competent patients resulted in the development of an immune response to TK-derived epitopes (25, 26). Once cytotoxic T lymphocytes (CTLs) against cells expressing HSV-TK are developed in patients, the transduced cells infused would be eradicated from the patient body in no time at all. Indeed, HSV-TK-expressing cells in our patients without any GvL effects had a very short time to survive in the patient's peripheral blood, which was measured by quantitative PCR (TaqMan PCR).

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Interestingly, the Italian group observed that patients who received infusions of transduced cells at the immunosuppressed condition, e.g. soon after stem cell transplantation, caused less development on such an immune response. These results suggest that TK-DLI is the most suitable therapy in the context of allo-SCT from partially mismatched or unrelated donors, where the risk of severe GvHD is particularly high, and patients are profound immunodeficient.

Another problem is weaker immune response of cultured cells against allo-antigens compared with that of primary lymphocytes. In general, *in vitro* culture to manipulate donor lymphocytes genetically impairs their immune functions (27, 28), which may explain why a few patients developed severe GvHD despite infusions of a large number of donor cells in our trial. An improved culture condition could preserve the T-cell repertoire and their immune functions (29).

### 6. CONCLUSIONS

The clinical trial confirmed the safety and therapeutic effects of the suicide-gene transduced lymphocytes for relapsed leukemia after allo-SCT. Furthermore, acute GvHD could be controlled by administration of GCV without any immunosuppressive drugs. However, rapid disappearance of transduced lymphocytes was also observed in patients without any clinical benefits. Since the suicide gene derived from viruses elicits immune responses in patients as a strong antigen, it is likely that CTLs against HSV-TK eradicated transduced cells soon after infusions. While an approach to the problem is to use the suicide genes of human origin instead (29, 30), an alternative is to combine TK-DLI with allo-SCT in which the patient's immune function is severely damaged to impair T cell priming against foreign antigens (24).

With further modifications including vector constructs (31, 32), culture conditions (33), and the timing of infusions, the suicide-gene strategy would offer the safe and effective immune cell therapy for patients with hematologic malignancies.

### 7. ACKNOWLEDGMENT

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**Abbreviations:** allo-SCT: allogeneic stem cell transplantation; GvL: graft versus leukemia; GvHD: graft versus host disease; DLI: donor lymphocyte infusion; GCV: ganciclovir; HSCs: hematopoietic stem cells; TBI: total-body irradiation; CTLs: cytotoxic T lymphocytes; CML: chronic myelogenous leukemia; TRM: transplant-related mortality; ATG: anti-thymocyte globulin; EDR: escalating dose regimen; HSV-TK: herpes simplex virus thymidine kinase; NGFR: nerve growth factor receptor; rIL-2: recombinant human interleukin-2

## **Gene and cell therapy for relapsed leukemia**

**Key Words:** Gene Therapy, Retroviral Vector, Suicide Gene, Graft-Versus-Leukemia, Donor Lymphocyte Infusion, Review

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## An immunotherapy approach with dendritic cells genetically modified to express the tumor-associated antigen, HER2

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**Abstract** Dendritic cells (DC), genetically modified to express ovalbumin by the retroviral vector GCDNsap, can elicit stronger anti-tumor immunity than those loaded with the peptides. To assess the clinical feasibility of the strategy, such DC were prepared by differentiation of hematopoietic progenitor cells transduced with the human epidermal growth factor receptor 2 (HER2). When inoculated in mice, the DC primed both HER2-specific cytotoxic T lymphocytes and type 1 T helper lymphocytes, resulting in production of HER2-specific antibody. Of importance is that the antibody mediated antibody-dependent cellular cytotoxicity and opsonization. The potent anti-tumor effects were also confirmed by results of experiments using HER2-transgenic mice. Inoculation of HER2-transduced DC resulted in longer disease-free survival of

treated mice that showed significant reduction of primary and metastatic tumors. Interestingly, footpad inoculation resulted in stronger anti-tumor effects compared to subcutaneous administration and induced higher levels of the HER2-specific antibody, suggesting that an important role of humoral immunity in anti-tumor effects for malignancies with membrane-type tumor-associated antigens (TAA). Taken together, vaccination of the TAA-transduced DC may represent a promising form of therapy for breast cancers expressing HER2.

**Keywords** Dendritic cells · Cancer · Tumor immunology · Immunotherapy · Vaccination

### Abbreviations

DC	Dendritic cell(s)
TAA	Tumor-associated antigen(s)
HER2	Human epidermal growth factor receptor 2
MHC	Major histocompatibility complex(es)
HPC	Hematopoietic progenitor cell(s)
OVA	Ovalbumin
CTL	Cytotoxic T lymphocyte(s)
Th1	Type 1 T helper
B6	C57BL/6N
FVB-Tg	FVB-Tg (MMTVneu) 202 Mul/J
tHER2 (M)	Truncated membrane form of HER2
PMC	Primary mammary carcinoma
PE	Phycoerythrin
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
EGFP	Enhanced green fluorescent protein
LN	Lymph node(s)
ADCC	Antibody-dependent cellular cytotoxicity

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## Introduction

Unique features of dendritic cells (DC), that is, they are capable of priming naïve T lymphocytes without additional exogenous adjuvants in the context of major histocompatibility complex (MHC) and immunostimulatory molecules have made them key players in cancer immunotherapy [2, 4, 11, 20, 34]. The establishment of sophisticated culture conditions allowing differentiation into mature DC from either hematopoietic progenitor cells (HPC) or peripheral monocytes has opened the way to clinical use of DC manipulated with loading of tumor-associated antigen (TAA)-derived peptides or tumor lysates in clinical vaccine trials for various cancers [1, 3, 24, 26, 39]. However, only a few cases have proven effective [7, 30], partly because of few available peptides restricted to the MHC class II molecules necessary to induce tumor-specific CD4<sup>+</sup> T lymphocytes. To overcome the problems, we have recently proposed that such DC be prepared by differentiation of HPC transduced with the cDNA of TAA using the silencing-resistant retroviral gene transfer vector, GCDNsap [25]. Indeed, DC that were differentiated from HPC transduced with an ovalbumin (OVA) cDNA induced more OVA-specific cytotoxic T lymphocytes (CTL) and type 1 T helper (Th1) lymphocytes in mice bearing OVA-expressing tumors than DC prepared by loading with the OVA peptides [25]. Of importance is that the generation of OVA-specific CD4<sup>+</sup> T lymphocytes induced production of antibody against OVA, which was not detected in mice inoculated with OVA peptide-pulsed DC [25].

Having verified the efficacy of the vaccine strategy for tumors bearing TAA with relatively strong immunogenicity, we set out to attempt to assess the capability of the transduced DC to elicit anti-tumor effects against tumors expressing TAA that we often encounter in clinical arena. Human epidermal growth factor receptor 2 (HER2), a member of the epidermal growth factor receptor tyrosine kinase family [5, 22], is frequently amplified and overexpressed in human cancers such as mammary, lung and ovary carcinomas [22, 32, 37]. In particular, it should be noted that the expression level of HER2 is significantly correlated with malignancy and poor prognosis [32]. Recent molecular biology has developed new and effective therapeutic methods as typified by molecular-targeted therapy. Among them, a humanized antibody against HER2, Trastuzumab, has proven effective in patients with advanced breast cancer [33]. On the contrary, clinical trials of vaccination using HER2 peptides or peptide-pulsed DC have reported disappointing clinical responses [8, 16]. In this study, we evaluated the potential of DC retrovirally modified to express the HER2 cDNA as a feasible treatment for breast cancer using a mouse model that develops mammary carcinoma spontaneously.

## Materials and methods

### Mice

C57BL/6N (B6) and FVB/N mice were purchased from Nihon Clea (Tokyo, Japan). FVB-Tg (MMTVneu) 202 Mul/J (FVB-Tg) mice expressing the rat *neu* under the control of the murine mammary tumor virus promoter [14] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed and bred under specific pathogen-free conditions at the Animal Resource Center of the University of Tsukuba. All experiments using mice were approved by the Institutional Review Committee and performed according to the guidelines of the University of Tsukuba.

### Retroviral vector construction and preparation

A 715 amino acids long product of the truncated HER2 membrane form void of the cytoplasmic kinase domain referred to as tHER2 (M) was amplified by PCR using the full-length HER2 cDNA (kindly provided by Dr. S. Shibata at Takeda Pharmaceutical Company, Osaka, Japan) as a template, 1.25 U of *PfuUltra* Hotstart DNA Polymerase (STRATAGENE, La Jolla, CA, USA) and the following primer set: 5'-CTGACGCGGCCGAGTGAGCACCA TG-3' (forward), and 5'-CTTCACCTTCCTCGAGCTCC GTCTATTTTCAGGAT-3' (reverse). The PCR product was once subcloned into pBlueScript (STRATAGENE) and a *NotI-XhoI* fragment containing the tHER2 (M) cDNA digested from the vector was inserted into the corresponding sites of the GCDNsap retroviral vector [35]. Infectious retroviral particles packaged in vesicular stomatitis virus G envelope (VSV-G) protein were generated by transduction into 293gpg [27] as described elsewhere [35]. The final titer was  $1 \times 10^7$  infectious units/ml on Jurkat cells [31]. The retrovirus was named GCDN/tHER2 (M).

### Cell cultures

EL4 [13] and its retrovirally transduced derivatives were cultured in RPMI 1640 with 10% fetal calf serum (HyClone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate (R10), and 50 µM 2-mercaptoethanol. Jurkat and SK-BR-3 [36] (kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University, Miyagi, Japan) were cultured in R10. 293gpg cells were maintained in a way as described [27]. Primary mammary tumors from female FVB-Tg mice were excised, minced and passed through a 70 µm diameter Cell Strainer (BD Biosciences, Falcon, San Jose, CA, USA) to generate primary mammary carcinoma (PMC) cells that were cultured in R10. All other culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Vaccination with HER2-transduced DC

DC pulsed with lysate of EL4 expressing tHER2 (M) (EL4/DN/EGFP & tHER2 (M)) and those transduced with tHER2 (M) were prepared as previously described [25, 26]. In a vaccine study,  $5 \times 10^5$  of lysate-pulsed or transduced DC were subcutaneously inoculated into the left flank of the B6 mice on Days -21, -14, -7, and  $5 \times 10^6$  EL4/DN/EGFP & tHER2 (M) cells were injected into the right flank on Day 0. Tumors were measured in two perpendicular diameters every 2 days for 2 weeks and represented as the Tumor Index. For FVB-Tg mice, tHER2 (M)-transduced DC were also inoculated into footpads of female mice. In a therapeutic setting,  $1 \times 10^6$  of the transduced DC were inoculated into footpads of tumor-bearing FVB-Tg mice three times. Stable disease periods after vaccination were defined as the number of days passed between the time of last tumor occurrence and the appearance of the next mass. Tumor-free periods, stable disease periods, the Tumor Index, and the number of tumors were recorded every 3 days up to the age of 300 days. The number of lung metastases was counted at 300 to 330 days.

### Flowcytometric analysis

Antibodies for surface antigens used are anti-c-ErbB2 (TA-1; Merck Biosciences, Calbiochem, San Diego, CA, USA), phycoerythrin (PE)-conjugated anti-mouse immunoglobulin (Ig) rabbit polyclonal F (ab')<sub>2</sub> (Dako, Produktionsvej, Denmark), CD11c (HL3; BD Bioscience, PharMingen), CD83 (Michel17, eBioscience, San Diego, CA, USA), biotinylated anti-mouse CD40 (HM40-3), CD86 (GL-1), I-A (M5/114.15.2) (eBioscience) and streptavidin-allophycocyanin (PharMingen). For intracellular staining, splenic CD4<sup>+</sup> T cells were treated with BD GolgiStop (PharMingen) and stained with fluorescein isothiocyanate-conjugated anti-mouse interleukin (IL)-2 (JES6-5H4; eBioscience) or PE-conjugated anti-mouse interferon (IFN)- $\gamma$  (XMG1.2; PharMingen). To detect anti-HER2 antibody in mouse sera, the HER2-positive human breast cancer cell line, SK-BR-3 cells were incubated with mouse serum and stained with PE-conjugated anti-mouse immunoglobulin (Ig) rabbit polyclonal F (ab')<sub>2</sub> (Dako), or biotinylated anti-mouse IgG<sub>1</sub> (A85-1), IgG<sub>2a</sub> (R19-15), IgG<sub>2b</sub> (R12-3), and IgG<sub>3</sub> (R40-82) and streptavidin-PE (PharMingen). Cells were analyzed with a FACSCalibur (BD Biosciences).

### Lysis spot assay

The assay was performed as previously described [25]. EL4 expressing enhanced green fluorescent protein (EGFP) or tHER2 (M) and EGFP were established by infection of EL4 with GCDN/EGFP [35] and GCDN/tHER2 (M), respectively. Splenic CD8<sup>+</sup> T cells obtained from B6 mice immunized with the tumor lysate-pulsed, untransduced or tHER2 (M)-transduced DC were co-cultured with  $1 \times 10^4$  target cells in Multiscreen-IP ELISpot plates (Millipore, Bedford, MA, USA) coated with anti-GFP antibody (Invitrogen Life Technologies, Molecular Probes, Carlsbad, CA, USA) at the indicated effector to target ratios [25].

51Cr-release assay

### 51Cr-release assay

Inguinal and popliteal lymph nodes (LN) isolated from the non-immunized or immunized FVB-Tg mice were treated with 1 mg/ml of collagenase D (Roche Diagnostics, Basel, Switzerland) at 37°C for 45 min followed by grinding and passage through a Cell Strainer (Falcon), and splenic CD8<sup>+</sup> T cells were isolated with mouse CD8-MicroBeads using Midi-MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). PMC cells ( $1 \times 10^4$ ) were labeled with 100  $\mu$ l of 2 mCi/ml [Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub>] (MP Biomedicals, Solon, OH, USA) and co-cultured at the indicated effector to target ratios in 96-Well Round Bottom TC-Treated Microplates (CORNING, Corning, NY, USA) at 37°C for 4 h with effector cells re-stimulated with tHER2 (M)-transduced DC. Chromium release in each well was determined with an auto well gamma system ARC-380 (ALOKA, Tokyo, Japan).

### Antibody-dependent cellular cytotoxicity (ADCC) assay

An ADCC activity induced by the immunized mouse sera was evaluated by JAM test [23]. EL4/DN/EGFP and EL4/DN/EGFP & tHER2 (M) cells as target cells were cultured in R10 containing 5  $\mu$ Ci/ml of [methyl-<sup>3</sup>H]-thymidine (TRA120; GE Healthcare Biosciences, Little Chalfont, UK) and stained with 1  $\mu$ l of mouse sera. Labeled target cells were co-cultured at the indicated effector to target ratios in 96-Well V Bottom TC-Treated Microplates (CORNING) at 37°C for 4 h with splenocytes that had been activated with 50 ng/ml mouse IL-2 (PharMingen) and 20 ng/ml mouse IL-12 p70 (eBioscience). DNA-bound [<sup>3</sup>H] radioactivity was measured with a liquid scintillation counter LS-6500 (Beckman Coulter, Fullerton, CA, USA).

### Opsonization assay

Macrophages were prepared as adherent cells in bone marrow cells that were cultured in R10 containing 10 ng/ml human macrophage colony-stimulating factor (R & D systems, Minneapolis, MN, USA) for 4 days. Irradiated EL4/DN/EGFP & tHER2 (M) cells as apoptotic cells were incubated with 1  $\mu$ l of the mouse sera and then co-cultured with macrophages for 1 to 3 h. The opsonic activity was estimated as the percentages of double Mac-1 (M1/70; PharMingen) and EGFP-positive cells determined by FACSCalibur (BD Biosciences).

### Migratory DC into draining LN

Five million EGFP-transduced DC were injected into the flank or footpads of FVB/N mice. At 72 h after inoculation, the number of DC that migrated into draining LN (inguinal and popliteal LN) was evaluated by flowcytometry and immunohistochemistry. In immunohistochemistry, de-paraffinized sections of draining LN were stained with a primary antibody solution containing 2 µg/ml of anti-GFP rabbit IgG (Molecular Probes) or isotype control (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Histofine Simple Stain Mouse MAX-PO (horseradish peroxidase-conjugated goat anti-rabbit polyclonal IgG Fab' polymer; Nichirei Bioscience, Tokyo, Japan). EGFP-expressing cells visualized with a DAKO ENVISION+ Kit/HRP (DAB) (Dako) were counted in randomly selected 20 microscope fields magnified by 200× and the average number per field was calculated.

### Adoptive transfer of splenocytes

CD4<sup>+</sup> or CD8<sup>+</sup> cells were removed from splenocytes of FVB-Tg mice that had been immunized with the transduced DC by the negative selection using anti-mouse CD4 (GK1.5) or CD8α (53-6.7) antibodies (eBioscience). Thirty millions of the CD4- or CD8-depleted splenocytes, or whole splenocytes were infused into non-immunized FVB-Tg mice through the tail vein and the tumor-free periods, Tumor Index, and the number of primary or metastatic tumors of the mice were recorded.

### Statistical analysis

Kaplan–Meier estimation with a Fischer-type generalized Wilcoxon method was used for statistical analysis of the differences between the tumor-free periods. The correlation between anti-HER2 antibodies titers and tumor-free periods, the number of tumor masses and lung metastases was evaluated with Spearman's rank-order correlation coefficient. All other statistical analyses were performed with the Mann–Whitney *U* test. A probability of less than 0.05 ( $P < 0.05$ ) was considered statistically significant.

## Results

### Characterization of DC genetically modified to express the truncated HER2

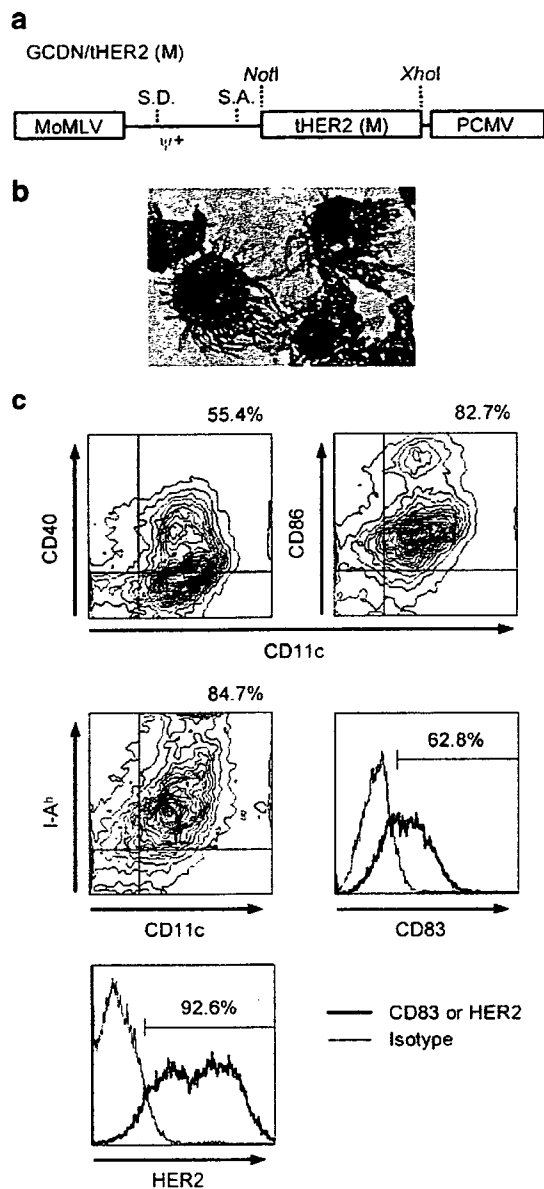
The primary end-point of the present study was to induce strong immune responses against HER2-expressing tumor through vaccination with the HER2-transduced DC. Therefore, to avoid potential negative effects of HER2

expression on DC, we used a truncated membrane form of the receptor (tHER2 (M)) devoid of the intracellular kinase domain that would be suitable for clinical applications. The structure of the gene-silencing-resistant retroviral vector GCDNsap encoding the tHER2 (M) cDNA is shown in Fig. 1a. The virus titer was approximately  $1 \times 10^7$  infectious units/ml on Jurkat cells. c-KIT<sup>+</sup>/lineage<sup>-</sup> cells isolated from B6 mouse bone marrow were transduced with the GCDN/tHER2 (M), expanded (31.2-fold  $\pm$  13.6), and then differentiated into DC (Fig. 1b). The cells expressed high levels of CD11c, CD40, CD86, MHC class II, and CD83 on their surface (Fig. 1c) and IL-12 p40 mRNA (data not shown), suggesting that they were mature DC. Approximately 5 to  $10 \times 10^6$  DC were prepared per mouse and more than 90% of the cells highly expressed HER2 (Fig. 1c).

### Anti-tumor immunity induced by tHER2 (M)-transduced DC

To assess the ability of the cells to induce anti-tumor effects,  $5 \times 10^5$  tHER2 (M)-transduced DC were subcutaneously inoculated into the left flank of B6 mice on Day -21, -14, and -7, following by graft of  $5 \times 10^6$  tHER2 (M)-expressing mouse lymphoma cell line EL4 cells (B6 background) into the right flank on Day 0. As the end of the observation periods, two out of four mice inoculated with transduced DC rejected the tumor graft completely and the other two showed significant inhibition of tumor progression (Fig. 2a). In contrast, mice inoculated with tumor lysate-pulsed DC as well as non-transduced ones showed little effects on the tumor growth. The results suggested that the transduced DC induced HER2-specific immunity, which was stronger than that observed in mice inoculated with conventional methods. To account for the difference of anti-tumor effects observed among three groups, the prevalence of HER2-specific CTL in those mice was compared by Lysis spot assay (Fig. 2b). While the number of spots observed in mice inoculated with tumor lysate-pulsed DC was similar to that of control mice, mice inoculated with transduced DC provided significantly more spots (Fig. 2b), suggesting that the transduced DC primed HER2-specific CTL more efficiently than did lysate-pulsed DC. In addition, mice immunized with transduced DC also generated HER2-specific Th1 cells that produced IL-2 and IFN-γ when co-cultured with tHER2 (M)-transduced DC, which were not detected in mice inoculated with lysate-pulsed DC (Fig. 2c). As expected, a high amount of total anti-HER2 antibody and IgG<sub>1</sub>, IgG<sub>2a</sub>, and IgG<sub>2b</sub> subclasses (left and right panels of Fig. 2d) were detected in sera from mice immunized with transduced DC after second vaccine. Plausible explanation for these results is that they could prime and activate both CD8<sup>+</sup> and CD4<sup>+</sup> T cells by presenting



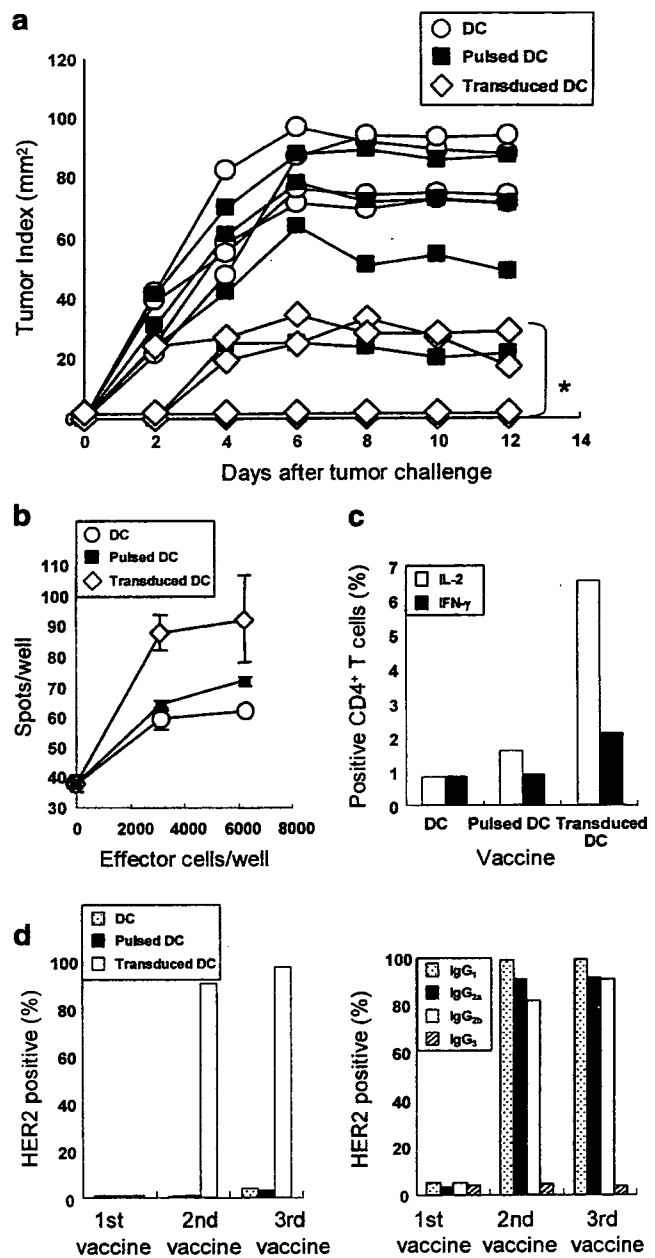


**Fig. 1** Structure of GCDN/tHER2 (M) and characters of tHER2 (M)-transduced DC. **a** Structure of GCDN/tHER2 (M). The vector contains the PCMV-derived long terminal repeat with intact splice donor and splice acceptor sequences. The tHER2 (M) cDNA was inserted between the *NotI* and the *XhoI* sites. Sequences present in the vector labeled as follows: *MoMLV* Moloney murine leukemia virus; *S.D.* splice donor;  $\Psi$ + packaging signal; *S.A.* splice acceptor; *PCMV* PCC4 cell-passaged myeloproliferative sarcoma virus. **b** Morphological appearances of transduced DC (May-Gruenwald-Giemsa staining). **c** Surface markers and transgene expression of transduced DC

HER2-derived epitope peptides in the context of MHC class I and II on their surface, respectively, resulting in establishment of HER2-specific CTL and Th1 cells.

Anti-tumor effects of the HER2-specific antibodies

Having observed that mice inoculated with transduced DC produced HER2-specific antibody, we next analyzed anti-



**Fig. 2** Induction of HER2-specific immunity by tHER2 (M)-transduced or tumor lysate-pulsed DC. **a** B6 mice inoculated with tumor lysate-pulsed, untransduced or tHER2 (M)-transduced DC were transplanted with EL4/DN/EGFP & tHER2 (M) cells on Day 0. Results of four mice per group are presented. \* $P < 0.05$  compared with the other groups. **b** CD8<sup>+</sup> T cells obtained from immunized mice were incubated with EL4/DN/EGFP & tHER2 (M) cells. The frequency of HER2-specific CTL was determined by Lysis spot assay. Representative results of one experiment out of independent three are shown. **c** CD4<sup>+</sup> T cells obtained from immunized mice were stained with anti-mouse IL-2 or IFN- $\gamma$  antibody intracellularly. **d** SK-BR-3 cells were stained with sera from mice immunized 1–3 times with each DC vaccine. The amount of total antibody (left) and IgG subclasses (right) against HER2 are shown as percentages of HER2-positive SK-BR-3 cells

tumor effects exerted by such antibody in several immunological assays. ADCC results in perforin- and granzyme-mediated destruction of antibody-coated target cells by

cells expressing Fc receptors, such as FcγRIII (CD16) on NK cells. When HER2-expressing EL4 cells were coated with sera obtained from mice immunized with transduced DC, they were efficiently lysed by splenocytes pre-stimulated with IL-2 and IL-12 (closed diamonds; Fig. 3a), while the same sera did not induce ADCC activity on the original EL4 cells that did not express HER2 (open diamonds). The specificity of the antibody against HER2-expressing cells was confirmed by a finding that HER2-expressing EL4 cells were not lysed by splenocytes when they were coated with sera of non-immunized mice (closed circles).

The antibody can also mediate phagocytosis by neutrophils and macrophages (through a mechanism known as opsonization). We found that sera from mice inoculated with transduced DC enhanced phagocytosis of irradiated HER2-expressing EL4 cells by macrophages (closed diamonds; Fig. 3b) whereas the phagocytosis was not detected for irradiated HER2-expressing EL4 cells coated with sera obtained from non-immunized mice (open circles). These results indicated that the anti-HER2 antibody in sera from immunized mice with transduced DC contributed to anti-tumor effects by ADCC activity and phagocytosis.

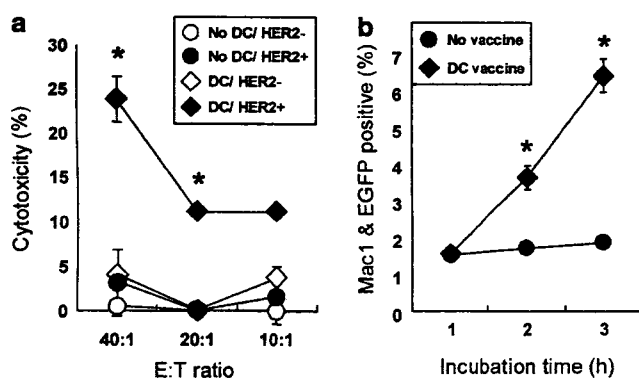
#### Anti-tumor effects of tHER2 (M)-transduced DC on spontaneous mammary carcinomas

To assess the feasibility of the HER2 (M)-transduced DC in a clinical setting where the vaccine would be conducted

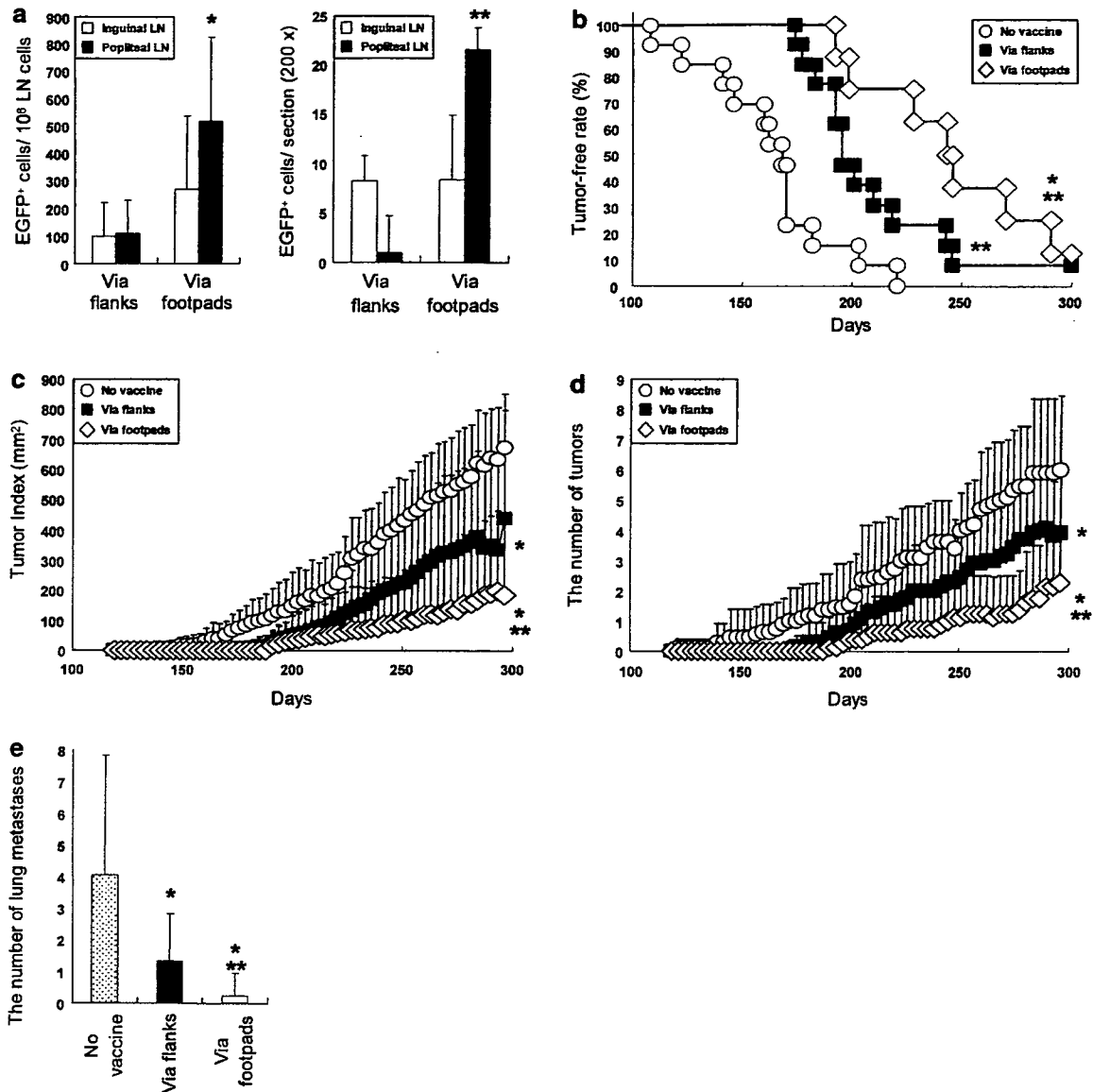
for cancer-bearing subjects, we applied the FVB/N-Tg (MMTVneu) 202 Mu1/J transgenic mouse model of breast cancer (FVB-Tg mice). Female FVB-Tg mice that spontaneously develop mammary carcinomas expressing the rat HER2 homolog are an ideal tool to assess the efficacy of cancer vaccines targeting HER2 [14]. c-KIT<sup>+</sup>/lineage<sup>-</sup> cells obtained from the parental mouse strain FVB/N were transduced with tHER2 (M), expanded (45.4-fold ±22.7) and induced to differentiate into mature DC. The phenotypes of these DC, including surface markers or cytokine expression patterns, the transduction efficiency and the yield of the DC were similar to those of DC obtained from B6 mice. Based on results of preliminary experiments using Balb/c mice that footpad inoculation of the transduced DC gave rise to higher amounts of HER2-specific antibody than did subcutaneous inoculation in flanks or intraperitoneal inoculation (unpublished observation), FVB-Tg mice were immunized with the transduced DC either at the flanks or footpads. Interestingly, the number of DC that migrated to their regional LN (popliteal and inguinal LN) by footpad inoculation was larger than that by subcutaneous inoculation (Fig. 4a). Consistent with the results, stronger anti-tumor effects in terms of the disease-free periods, tumor size, the number of primary and lung metastatic tumors were observed in mice with footpad inoculation (Fig. 4b–e), suggesting that the footpad inoculation induced potent HER2-specific immune responses by facilitating migration of the infused DC into their regional LN.

#### Comparison of immune responses induced by inoculation at flanks and footpads

To determine the main contributor to the difference in anti-tumor effects dependent on the route of inoculation, cellular and humoral immune responses against HER2 induced by inoculation of the tHER2 (M)-transduced DC either at flanks and footpads were further analyzed. When the HER2-specific CTL activity of CD8<sup>+</sup> T cells in the spleen and draining LN cells of immunized mice was measured by co-culture with PMC cells that developed in untreated FVB-Tg mice, both mice inoculated via either route showed similar CTL activities (Fig. 5a). Furthermore, the number of HER2-specific Th1 cells in both mice was not significantly different (Fig. 5b). However, footpad inoculation produced a significantly higher amount of all anti-HER2 IgG subclasses than did subcutaneous inoculation at the flanks (Fig. 5c). Interestingly, the subcutaneous inoculation produced a high amount of HER2-specific IgG<sub>1</sub> and little, if any, other HER2-specific IgG subclasses. In agreement with results of experiments using B6 mice (Fig. 3a), the anti-HER2 antibody, especially that in footpad inoculation, exerted the strong ADCC activity against HER2-expressing



**Fig. 3** Involvement of antibody against HER2 in anti-tumor effects. **a** EL4/DN (open) or EL4/DN/EGFP & tHER2 (M) cells (closed) were incubated with sera from B6 mice vaccinated with or without tHER2 (M)-transduced DC (diamonds or circles, respectively) and co-cultured with activated splenocytes. \* $P < 0.05$  compared with the other groups. **b** Apoptotic EL4/DN/EGFP & tHER2 (M) cells stained with sera from mice immunized with or without transduced DC (diamonds or circles, respectively) were co-cultured with macrophages. The opsonic activity was shown as the percentages of EGFP-positive Mac-1<sup>+</sup> cells. \* $P < 0.01$  compared with non-treated mice. Results shown are representative in three independent experiments



**Fig. 4** Anti-tumor efficacy of the transduced DC for the FVB-Tg mice. **a** The number of DC migrating into draining LN was calculated by flowcytometry (left) and immunohistochemistry (right). \**P* < 0.05 compared with the other groups. \*\**P* < 0.005 compared with the other groups. **b** Tumor-free periods of untreated FVB-Tg mice (*n* = 13) or inoculated with tHER2 (M)-transduced DC via flanks (*n* = 13) or foot-

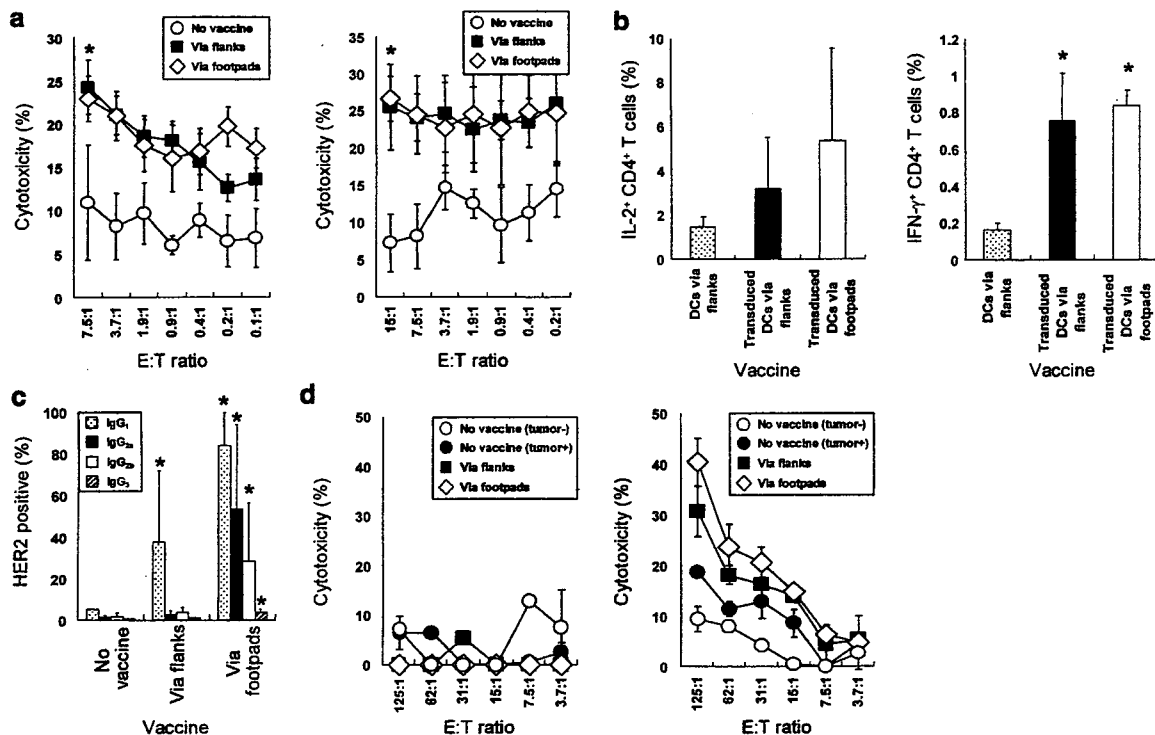
pads (*n* = 8). \**P* < 0.05 compared with via flanks, \*\**P* < 0.005 compared with no vaccine. Tumor Index (**c**), the number of primary tumors (**d**), and lung metastases (**e**) in mice left untreated or inoculated with the transduced DC via flanks or footpads are shown. \**P* < 0.05, flanks versus no vaccine, and footpads versus flanks, and \*\**P* < 0.01, footpads versus no vaccine in (**c**) (**d**) and (**e**)

tumors in an antigen-specific manner (Fig. 5d), suggesting that the different levels of humoral immunity against HER2 induced by the inoculation route mainly contributed to the difference of anti-tumor effects observed between mice.

**Anti-tumor effects of humoral immunity against spontaneous mammary carcinomas**

To assess a role of humoral immunity in anti-tumor effects against spontaneous mammary carcinomas in FVB-Tg

mice, we infused CD4<sup>+</sup>-depleted, CD8<sup>+</sup>-depleted or whole splenocytes prepared from the spleen of mice that had been vaccinated with tHER2 (M)-transduced DC into non-immunized FVB-Tg mice. In contrast to mice infused with whole splenocytes where the potent anti-tumor effects were observed comparable to those observed in mice inoculated with transduced DC, neither group infused with CD4- nor CD8-depleted splenocytes showed anti-tumor effects in terms of the tumor-free periods, tumor progression, and the number of tumor masses (*P* < 0.05, Fig. 6a–d). Furthermore, the level of HER2-specific IgG<sub>2a</sub> antibody, not a total



**Fig. 5** Elicitation of HER2-specific immune responses dependent on inoculation pathways. **a** Splenic CD8<sup>+</sup> T cells (*left*) or LN cells (*right*) from FVB-Tg mice left untreated or inoculated with tHER2 (M)-transduced DC via flanks or footpads were co-cultured with PMC cells. Results of four independent experiments are shown. \* $P < 0.05$ , flanks, footpads versus no vaccine. **b** CD4<sup>+</sup> T cells from mice immunized the indicated DC vaccine were stained with anti-mouse IL-2 (*left*) or IFN-

$\gamma$  antibody (*right*) intracellularly. Results of three independent experiments are shown. \* $P < 0.05$  compared with DC via flanks. **c** The amount of antibodies against HER2 was estimated by the percentages of HER2-positive SK-BR-3. \* $P < 0.01$ , flanks versus no vaccine, and footpads versus the others. **d** EL4/DN (*left*) or EL4/DN/EGFP & tHER2 (M) cells (*right*) were incubated with sera from immunized mice and co-cultured with activated splenocytes

amount of HER2-specific antibody, positively correlated with anti-tumor effects represented as tumor-free periods, the number of tumor masses and lung metastases (Fig. 7a, b). Taken together, it is likely that the potent anti-tumor effects observed here were sustained by both cellular and humoral immunity against HER2.

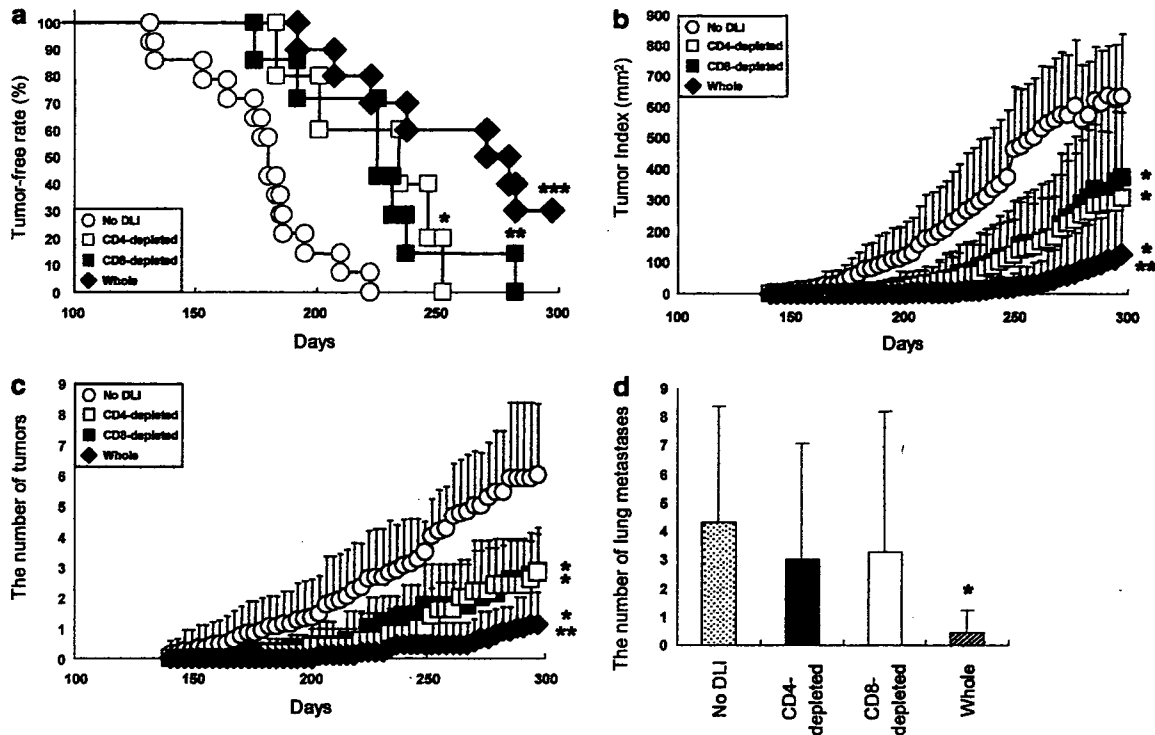
#### Anti-tumor effects of transduced DC in a therapeutic setting

Finally, to evaluate anti-tumor effects of vaccine with tHER2 (M)-transduced DC in a therapeutic setting, FVB-Tg mice that had already developed mammary tumors were inoculated three times with  $1 \times 10^6$  transduced DC via footpad route. In the result, 4 out of 41 tumors present in the mice at the vaccine were completely eradicated and six partially regressed (Fig. 8a). Of note, the eradicated tumors did not relapse over 60 days of observation. Although nine tumors kept increasing in size, 22 tumors remained stable. Compared with untreated mice, mice inoculated with transduced DC showed significantly fewer tumors and longer stable disease periods (Fig. 8b, c). The results suggested that vaccine with tHER2 (M)-transduced DC elicited potent anti-tumor effects against pre-existing mammary carcinomas in a situation very similar to clinical arena.

#### Discussion

In the present study, we demonstrate the potent anti-tumor effects of the tHER2 (M)-transduced DC in vivo with induction of both cellular and humoral immunity against tumors expressing HER2. Of importance is that the effects were confirmed by experiments using FVB-Tg mice in which multiple mammary carcinomas expressing HER2 developed spontaneously. Mice inoculated with transduced DC showed less primary and metastatic tumors and longer disease-free periods compared to non-treated mice. In addition to these observations, we also observed the superiority of footpad inoculation over subcutaneous one in facilitation of DC migration into their draining LN, resulting in induction of strong humoral immune responses. Finally, the vaccine strategy with the transduced DC via footpad route induced strong immune responses against the tumors even in mice with pre-existing mammary carcinomas.

Although DC genetically modified to express TAA by a retroviral vector are likely to be more beneficial than those pulsed with TAA-derived epitope peptides or tumor lysates because of continuous and stable presentation of the TAA through the both MHC class I and II on their surfaces



**Fig. 6** Adoptive transfer of splenocytes from immunized FVB-Tg mice. **a** Tumor-free periods of untreated FVB-Tg mice (No DLI, open circles,  $n = 14$ ), or mice infused with whole (closed diamonds), CD4- (open squares) or CD8-depleted (closed squares) splenocytes ( $n = 10, 5, 7$ , respectively). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.005$  compared

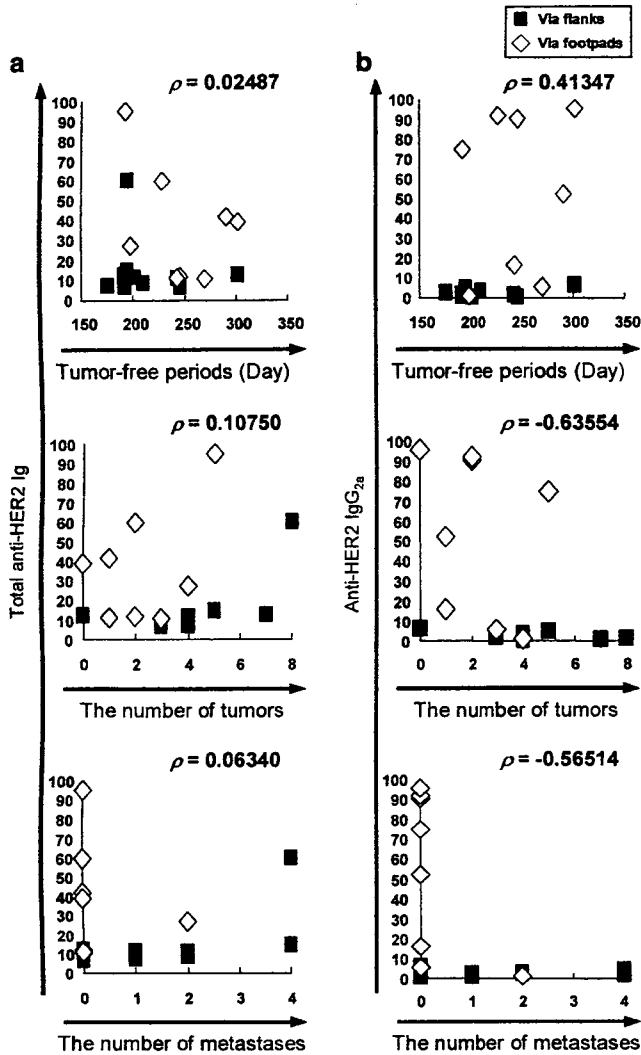
with untreated mice. Tumor Index (**b**), the number of primary tumors (**c**), and lung metastases (**d**) in mice with or without adoptive transfer. \* $P < 0.05$ , CD4-, CD8-depleted compared to No DLI or Whole, and \*\* $P < 0.005$ , Whole compared to No DLI in (**b**) and (**c**). \* $P < 0.05$  compared to other groups in (**d**)

[12, 15, 17, 18, 29, 38], no clinical trials using such DC have been reported, partly because of low transduction efficiency and gene silencing of conventional retroviral vectors when used for HPC and mature DC [17, 19, 38]. We have developed the gene-silencing-resistant retroviral vector, GCDNsap [35] and demonstrated the ability of the vector to express the transgene in mature DC [25]. Indeed, DC transduced with OVA cDNA using the retroviral vector elicited potent anti-tumor immune responses against tumors expressing OVA [25] similar to what observed in the present study for HER2 antigen, suggesting the general versatility of the strategy described here in term of TAA-derived epitope presentation.

While the present study demonstrated that vaccination with tHER2 (M)-transduced DC was superior to that with tumor lysate-pulsed DC, it also showed the importance of vaccine sites, with footpad inoculation providing more potent anti-tumor effects than subcutaneous immunization at the flanks by producing higher levels of anti-HER2 antibody, especially IgG<sub>2a</sub> subclass. The result may suggest that both inoculation pathways are sufficient to fully activate HER2-specific CTL, resulting in the maximal plateau of anti-HER2 cellular immunity in vivo. On the contrary, anti-HER2 humoral immunity remains insufficient in the

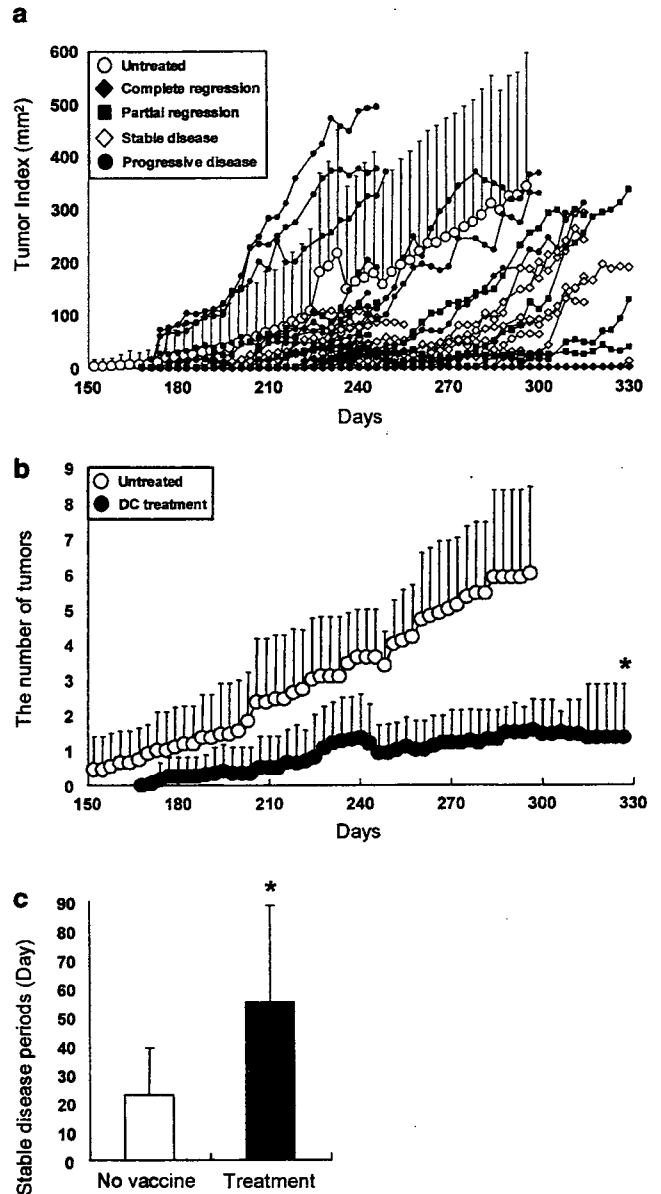
case of subcutaneous inoculation that is unlikely to be a proper route for DC migration into their draining LN. Although the reason why subcutaneous inoculation at the flanks induced less humoral immunity while eliciting strong cellular immunity comparable to footpad inoculation should be reconciled by additional experiments, effective anti-tumor effects against tumors expressing TAA on their surfaces may necessitate potent humoral immunity against the tumors as demonstrated in clinical studies using Trastuzumab (humanized antibody against HER2) for breast cancer patients.

It should be noted that the HER2 cDNA used for treatment of FVB-Tg mice expressing rat HER2 homolog is the human HER2/ErbB2. Unfortunately, we cannot deny that use of the xenoantigen may jeopardize the promising results observed in the present study, but it is also true that orthologous proteins for “self” antigens is commonly used to break self-tolerance due to its higher immunogenicity [9, 10, 21, 28]. Therefore, use of the orthologous HER2 antigen derived from other species except for human, such as mouse or rat and direct injection of DC into patient’s axillary or inguinal LN with ultrasound echo guidance may be worth considering to enhance anti-tumor immunity in clinical trials.



**Fig. 7** The correlation of anti-HER2 antibodies with prognoses of FVB-Tg. Correlation of total anti-HER2 Ig (a) or IgG<sub>2a</sub> (b) with tumor-free period (*upper*) and the number of primary tumors (*middle*) or lung metastases (*lower*) was evaluated with Spearman's rank-order correlation coefficient and represents  $\rho$  values

The present study showed promising results from the use of transduced DC-mediated anti-tumor immunity against pre-existing tumors in FVB-Tg mice, which encourages us to take a step to apply the strategy in a clinical setting. Furthermore, considering that the considerable number of clinical trials for primary immunodeficiency where the patient CD34<sup>+</sup> cells are genetically modified by retroviral vectors has proven beneficial [6], our strategy that patient CD34<sup>+</sup> cells transduced with HER2 by GCDNsap are differentiated into mature DC in vitro followed by infusion into patients' LN directly has sufficient possibility for clinical applications. In a situation where few vaccine trials using HER2 peptides or peptide-pulsed DC proved beneficial [7, 8, 16], the HER2-transduced DC described here may present attractive and promising therapy for mammary carcinomas.



**Fig. 8** Treatment with the transduced DC for tumor-bearing FVB-Tg mice. **a** Kinetics of tumor progression of tumor-bearing FVB-Tg mice inoculated with transduced DC ( $n = 20$ ) or without treatments ( $n = 11$ ) are shown. These mice are classified as complete regression, partial regression, stable disease, and progressive disease. The number of tumors (**b**) and stable disease periods (**c**) in mice treated with transduced DC or untreated mice. \* $P < 0.005$  compared with untreated

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# BASIC–LIVER, PANCREAS, AND BILIARY TRACT

## Enhanced Self-Renewal Capability in Hepatic Stem/Progenitor Cells Drives Cancer Initiation

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**Background & Aims:** Transformed hematopoietic stem/progenitor cells with an enhanced or acquired self-renewal capability function as leukemic stem cells. In a variety of solid cancers, stem/progenitor cells could be also targets of carcinogenesis. However, it remains unclear whether disruption of stem cell function directly contributes to cancer initiation. We sought to elucidate the mechanisms of self-renewal in hepatic stem/progenitor cells and the relation between stem cell function and hepatocarcinogenesis. **Methods:** Functional analyses of polycomb-group protein Bmi1 and Wnt/ $\beta$ -catenin, the molecules that are responsible for the self-renewal capability of many types of stem cells, were conducted in c-Kit<sup>+</sup>CD29<sup>+</sup>CD49f<sup>+/low</sup>CD45<sup>-</sup>Ter-119<sup>-</sup> hepatic stem/progenitor cells using retrovirus- or lentivirus-mediated gene transfer. The tumorigenicity of these cells transduced with the indicated retroviruses was also assessed by transplantation into nonobese diabetic/severe combined immunodeficient mice. **Results:** Forced expression of Bmi1 and constitutively active  $\beta$ -catenin mutant similarly promoted the self-renewal of hepatic stem/progenitor cells. The transplantation of Bmi1- or  $\beta$ -catenin-transduced cells clonally expanded from single hepatic stem/progenitor cells produced tumors, which exhibited the histologic features of combined hepatocellular and cholangiocarcinoma. **Conclusions:** These observations imply that the dysregulated self-renewal of hepatic stem/progenitor cells serves as an early event in hepatocarcinogenesis, and they highlight the important roles of Bmi1 and the Wnt/ $\beta$ -catenin pathway in regulating the self-renewal of normal or cancer stem cells in liver.

Current advances in stem cell biology-based technologies such as fluorescent-activated cell sorting (FACS) and in vivo transplantation models now allow the identification and characterization of stem cells in a variety of solid tissues and organs. In liver, hepatoblasts in the fetal liver and oval cells in the adult liver are thought to be hepatic stem/progenitor cells,<sup>1,2</sup> but the phenotypes of these cells have long been unknown. Recently, we demonstrated that the FACS sorting of c-Kit<sup>+</sup>CD29<sup>+</sup>CD49f<sup>+</sup>CD45<sup>-</sup>Ter-119<sup>-</sup> cells achieves an efficient enrichment of hepatic colony-forming units in culture (H-CFU-Cs), giving rise to colonies containing >100 cells in culture for 5 days.<sup>3,4</sup> Some of the H-CFU-Cs exhibit self-renewal capability accompanied by the potential for multilineage differentiation for >6 months and are considered putative stem/progenitor cells in fetal liver.

Stem cells comprise an extremely minor population that possesses the capabilities of extensive self-renewal, differentiation into progenies, and repair of damaged tissue.<sup>5</sup> Several molecules and pathways have been determined to play critical roles in the homeostatic self-re-

**Abbreviations used in this paper:** CC, cholangiocarcinoma; cHCC-CC, combined hepatocellular and cholangiocarcinoma; CK7, cytokeratin 7; EGFP, enhanced green fluorescent protein; FACS, fluorescent-activated cell sorting; HCC, hepatocellular carcinoma; H-CFU-C, hepatic colony-forming unit in culture; HPRT, hypoxanthine phosphoribosyl transferase; HSC, hematopoietic stem cell; IgG, immunoglobulin G; NOD/SCID, non-obese diabetic/severe combined immunodeficient; NSC, neural stem cell; PCR, polymerase chain reaction; RT, reverse transcriptase; shRNA, short hairpin RNA.

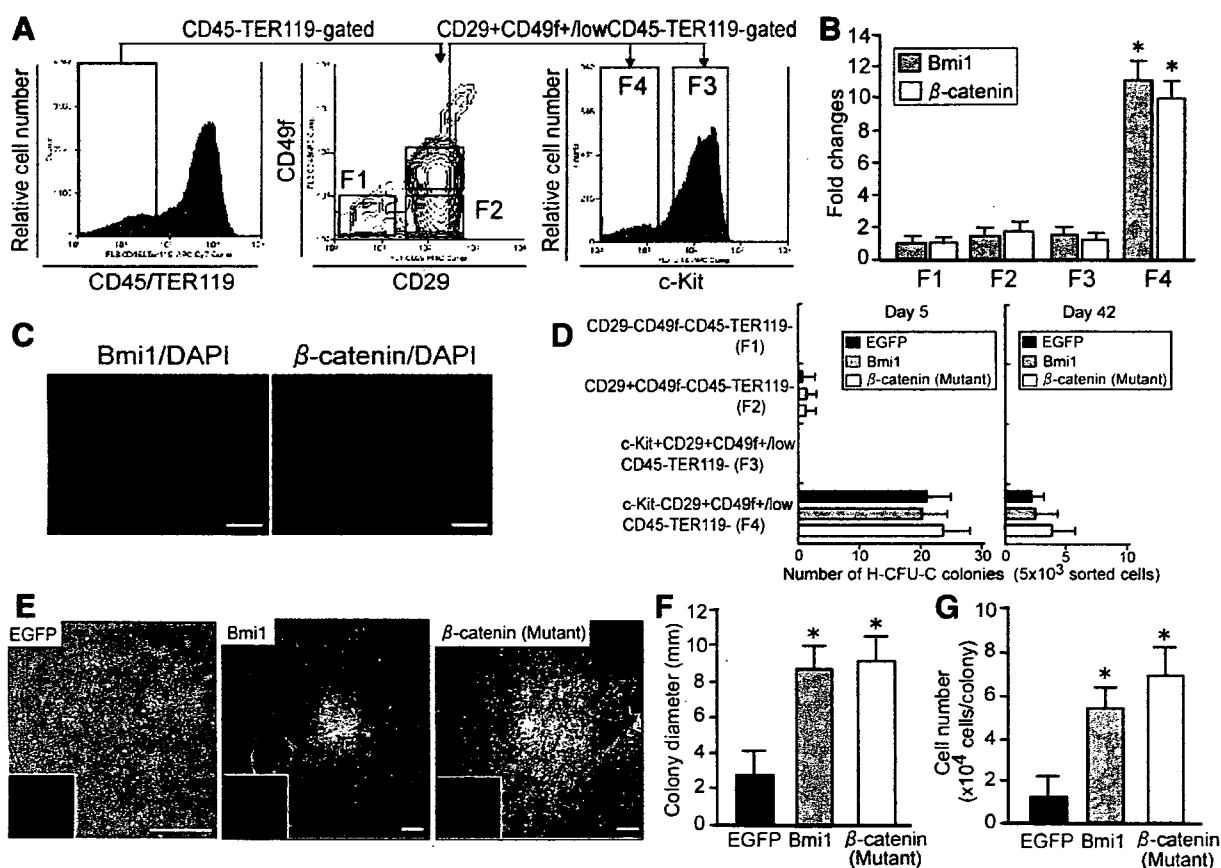
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newal of somatic stem cells, including hematopoietic stem cells (HSCs) and neural stem cells (NSCs).<sup>6,7</sup> However, little is known about the mechanisms responsible for the self-renewal capability of hepatic stem cells.

We and others reported that the polycomb-group gene *Bmi1* is a critical determinant of the self-renewal capacity of HSCs, based on gain-of-function and loss-of-function analyses.<sup>8,9</sup> Moreover, it was reported that *Bmi1* is essential for the self-renewal of NSCs and expansion of early neural progenitors.<sup>10,11</sup> These observations indicate that *Bmi1* functions as a universal molecule for stem cell

self-renewal. The *Wnt/β-catenin* pathway is also involved in the maintenance of a variety of stem cells such as HSCs, NSCs, and epidermal stem cells.<sup>12</sup> In liver, the *Wnt/β-catenin* pathway is tightly associated with hepatocyte proliferation in development, regeneration, and tumor growth,<sup>13-16</sup> but its role in stem/progenitor cells remains unclear.

Signaling pathways that regulate the self-renewal of stem cells are important drivers of cell proliferation and survival and are frequently relevant to carcinogenesis when disrupted by mutations. *Bmi1* plays a crucial role in



**Figure 1.** Isolation of hepatic stem/progenitor cells from murine fetal livers (embryonic day 13.5) and transduction of *Bmi1* or constitutively active  $\beta$ -catenin. (A) c-Kit<sup>+</sup>CD29<sup>+</sup>CD49f<sup>+</sup>lowCD45<sup>-</sup>Ter-119<sup>-</sup> cells (fraction 4; F4), a major cell fraction which contains H-CFU-Cs, represented approximately 2.0% of all fetal liver cells. (B) Real-time RT-PCR analyses showed that the expression of *Bmi1* and  $\beta$ -catenin was specifically up-regulated in c-Kit<sup>+</sup>CD29<sup>+</sup>CD49f<sup>+</sup>lowCD45<sup>-</sup>Ter-119<sup>-</sup> cells (F4) 11.2-fold and 10.0-fold, respectively, compared with CD29<sup>+</sup>CD49f<sup>+</sup>CD45<sup>-</sup>Ter-119<sup>-</sup> cells (F1). \*Statistically significant ( $P < .05$ ). (C) Immunocytochemical analyses revealed the nuclear localization of *Bmi1* (left panel) and the nuclear/cytoplasmic localization of  $\beta$ -catenin (right panel) in nearly 100% and 60% of freshly isolated c-Kit<sup>+</sup>CD29<sup>+</sup>CD49f<sup>+</sup>lowCD45<sup>-</sup>Ter-119<sup>-</sup> cells (F4), respectively (scale bar, 5  $\mu$ m). (D) Cells from each fraction was transduced with EGFP, *Bmi1*, or mutant  $\beta$ -catenin, and allowed to form colonies. c-Kit<sup>+</sup>CD29<sup>+</sup>CD49f<sup>+</sup>lowCD45<sup>-</sup>Ter-119<sup>-</sup> cells (F4), but not the other fractions (F1-3), efficiently gave rise to colonies, some of which kept growing for 42 days. The number of colonies generated from  $5 \times 10^3$  F4 cells transduced with EGFP, *Bmi1*, and mutant  $\beta$ -catenin was  $21.5 \pm 4.5$ ,  $20.0 \pm 4.1$ , and  $23.3 \pm 5.0$ , respectively at day 5 of culture. The number of colonies that kept growing for 42 days was  $2.2 \pm 1.0$ ,  $2.4 \pm 1.9$ , and  $3.8 \pm 1.5$ , respectively. These data were obtained from 6 independent experiments. (E) Transduction of *Bmi1* or mutant  $\beta$ -catenin into H-CFU-Cs gave rise to larger colonies compared with the control when cultured for 42 days, which showed a "pile-up" appearance in the central area. Note that the scale bar is different between the control and the other colonies (scale bar, 1 mm). (F) The diameter of colonies expressing EGFP, *Bmi1*, and mutant  $\beta$ -catenin at day 42 of culture was  $2.6 \pm 1.4$  mm,  $8.3 \pm 1.5$  mm, and  $8.6 \pm 1.7$  mm, respectively. \*Statistically significant ( $P < .05$ ). (G) The number of cells in H-CFU-C colonies expressing EGFP, *Bmi1*, and mutant  $\beta$ -catenin at day 42 of culture was  $1.1 \pm 1.0 \times 10^4$ ,  $5.4 \pm 0.9 \times 10^4$ , and  $6.2 \pm 1.5 \times 10^4$ , respectively. \*Statistically significant ( $P < .05$ ).

the occurrence of hematologic malignancies and brain tumors.<sup>6,7</sup> The canonical Wnt pathway is also responsible for a wide variety of malignancies.<sup>17,18</sup> Notably, both increased expression of the *Bmi1* gene and activation of the Wnt/ $\beta$ -catenin pathway are frequently observed in liver cancers such as hepatocellular carcinoma (HCC).<sup>19,20</sup> Accumulating evidence now implies that self-renewing stem cells or progenitor cells that reacquire the capacity to self-renew might be one of the major origins of cancer.<sup>21</sup> In fact, subpopulations that exhibit stem/progenitor activities in prostate and mammary glands have been shown to be targets of neoplastic transformation.<sup>22,23</sup> Furthermore, the involvement of facultative hepatic stem/progenitor cells in hepatocarcinogenesis was also shown in a mouse model.<sup>24</sup> It is of paramount importance to elucidate the mechanisms underlying hepatocarcinogenesis derived from stem/progenitor cells, because persons with HCC with a similar gene expression profile to hepatic stem/progenitor cells have a poor prognosis.<sup>25</sup>

In the present study, we conducted both gain-of-function and loss-of-function analyses to investigate the impact of the self-renewal signals, *Bmi1* and the Wnt/ $\beta$ -catenin pathway, on purified hepatic stem/progenitor cells. We also prospectively examined the process of neoplastic transformation of hepatic stem/progenitor cells, providing solid evidence of hepatocarcinogenesis that targets hepatic stem/progenitor cells.

## Materials and Methods

### Mice

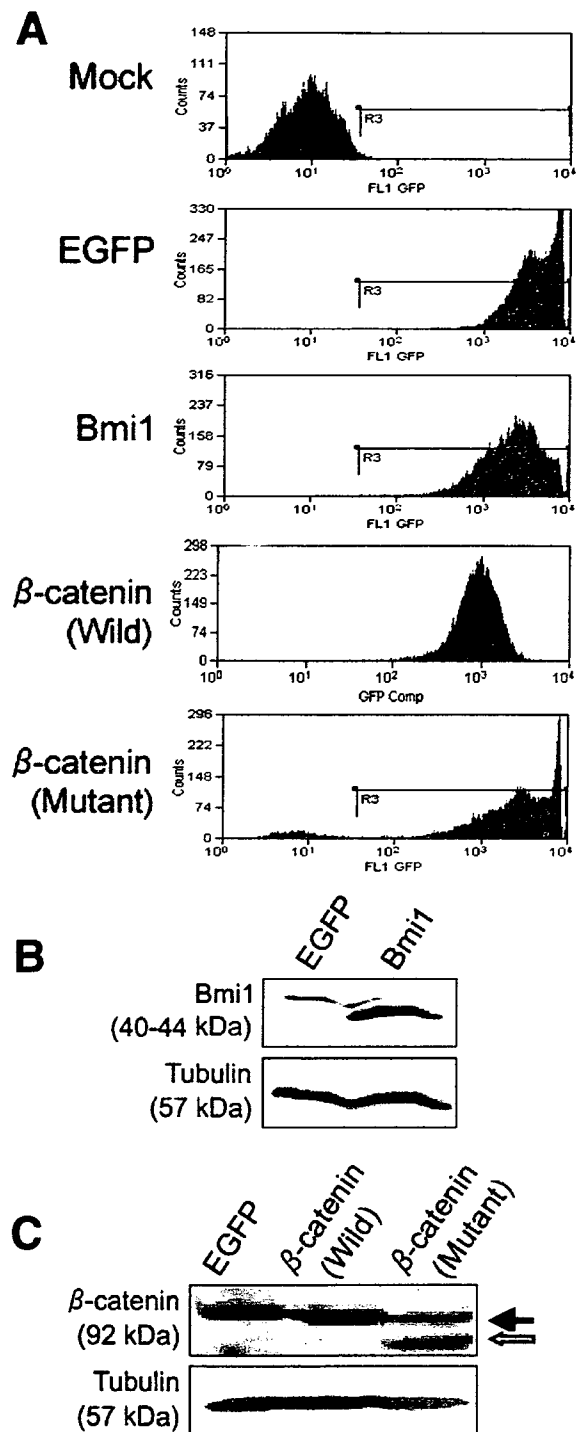
C57BL/6 and nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Japan SLC (Hamamatsu, Japan) and Sankyo Laboratory Co Ltd (Tsukuba, Japan), respectively. They were bred and maintained in accordance with our institutional guidelines for use of laboratory animals.

### Purification of Hepatic Stem/Progenitor Cells

Suspended liver cells were obtained from embryonic day 13.5 C57BL/6 fetal mice as described previously.<sup>3,4</sup> Briefly, dissociated liver cells were stained with biotinylated anti-CD45 and Ter-119 monoclonal antibodies, fluorescein isothiocyanate-conjugated anti-CD29 antibody, phycoerythrin-conjugated anti-CD49f antibody and allophycocyanin-conjugated anti-c-Kit antibody. Cells positive for the biotinylated antibodies were detected with streptavidin-labeled allophycocyanin-Cy7 (all from BD Pharmingen, San Diego, CA). The cells were analyzed and sorted using MoFlo (DakoCytomation, Fort Collins, CO).

### Real-Time RT-PCR

Total RNA from freshly sorted cells or colonies was extracted using Isogen reagent (Nippon Gene, Toyama, Japan). RNA (1  $\mu$ g) was reverse-transcribed to



**Figure 2.** The colonies at day 42 of culture were replated, then subjected to FACS and Western blot analyses. (A) The EGFP positivity in cells expressing EGFP, *Bmi1*, wild-type  $\beta$ -catenin, and mutant  $\beta$ -catenin was 99.9%, 99.9%, 99.9%, and 97.0%, respectively. (B) Western blot analysis showed an increased level of *Bmi1* in *Bmi1*-transduced cells. (C) Retroviral transduction of wild-type and mutant  $\beta$ -catenin resulted in enhanced expression of  $\beta$ -catenin (arrow) and ectopic expression of deletion  $\beta$ -catenin mutant (open arrow), respectively.

cDNA using MuLV reverse transcriptase (RT; Applied Biosystems, Foster City, CA). PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The Taqman probe and primers for *Bmi1* (assay ID, Mm00776122\_gH),  $\beta$ -catenin (assay ID, Mm00483033\_m1), and *hypoxanthine phosphoribosyl transferase* (*HPRT*; assay ID, Mm0000446968\_m1) were obtained from TaqMan Gene Expression Assays (Applied Biosystems). PCR for *Axin* was also performed using the Universal ProbeLibrary probe (catalog no, 04685105001; Roche Diagnostics, Mannheim, Germany) and the following primer set: forward, 5'-CAGCCCCTAATCCCC-TGA-3'; reverse, 5'-GCATGACTGCCTGCACATAC-3'.

### Immunocytochemistry

Freshly isolated c-Kit<sup>-</sup>CD29<sup>+</sup>CD49f<sup>+/low</sup>CD45<sup>-</sup>Ter-119<sup>-</sup> cells were placed on poly-L-lysine-coated slide glasses. After fixation with 2% paraformaldehyde and blocking in 10% goat serum, cells were incubated with a primary antibody, anti-mouse *Bmi1* (F6; Upstate Biotechnology, Lake Placid, NY) or anti-mouse  $\beta$ -catenin (clone14; BD) at a dilution of 1:200 for 12 hours at 4°C. The cells then were washed and incubated with Alexa-555-conjugated goat anti-rabbit immunoglobulin G (IgG; Molecular Probes, Eugene, OR) at a dilution of 1:500 for 2 hours at room temperature.

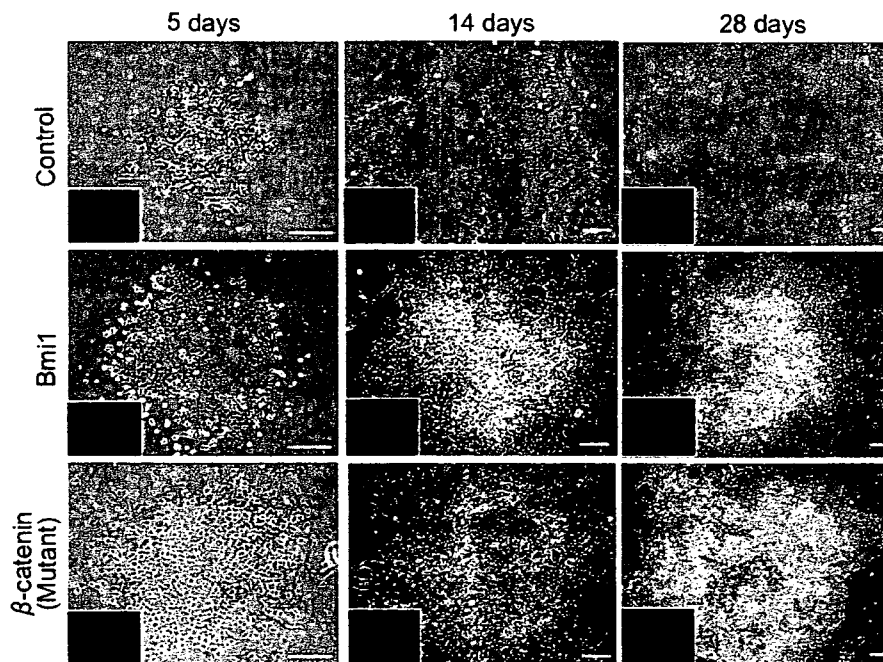
### Virus Production and Transduction

cDNA of the constitutively active form of murine  $\beta$ -catenin lacking exon 3 was constructed as described previously.<sup>26</sup> cDNAs of *Bmi1*, wild-type  $\beta$ -catenin, mutant  $\beta$ -catenin, *Axin* (a gift from Dr. Yukiko Gotoh), *cyclin D1*

(a gift from Dr. Yukio Nakamura), and *CDK4* (a gift from Dr. Itaru Matsumura) were cloned into a site upstream of IRES-enhanced green fluorescent protein (EGFP) in the pGCDNsam retroviral vector. Lentiviral vectors (CS-H1-shRNA-EF-1 $\alpha$ -EGFP) expressing short hairpin RNA (shRNA) against murine *Bmi1* (target sequence: TAAAG-GATTACTACACGCTAATG) and luciferase were constructed. Recombinant retroviruses and lentiviruses were produced as described elsewhere.<sup>8,27</sup> Sorted cells, which had been plated on collagen type IV-coated 6-well plates (Becton Dickinson, Oxford, United Kingdom) at  $5 \times 10^3$  cells/well, were then transduced with viruses in the presence of protamine sulfate (10  $\mu$ g/mL; Sigma-Aldrich, St. Louis, MO).

### In Vitro Colony Assay

The colonies derived from c-Kit<sup>-</sup>CD29<sup>+</sup>CD49f<sup>+/low</sup>CD45<sup>-</sup>Ter-119<sup>-</sup> cells were subcultured at day 42 of culture. They were clone sorted onto collagen type IV-coated 96-well microtiter plates (Becton Dickinson), and the number of H-CFU-Cs, which give rise to colonies consisting of >100 cells at day 5 of culture, were counted.<sup>3,4</sup> For immunocytochemical analyses, methanol-fixed cells were stained with rabbit antialbumin (Biogenesis, Poole, United Kingdom) and mouse anti-cytokeratin 7 (CK7) (DakoCytomation), followed by incubation with Alexa-555-conjugated goat anti-rabbit IgG (Molecular Probes) and Alexa-488-conjugated goat anti-mouse IgG (Molecular Probes), respectively. The mRNA expression of hepatocyte- and cholangiocyte-lineage markers in H-CFU-C colonies was examined by RT-PCR using primer sets and PCR conditions described



**Figure 3.** Sequential changes of secondary colonies showing features similar to those of the primary colonies. Colonies at day 42 in Figure 1E were replated. The secondary colonies expressing *Bmi1* or mutant  $\beta$ -catenin showed a similar appearance to the control at day 5 but exhibited a pile up appearance at days 14 and 28 (Scale bar, 100  $\mu$ m).