英国エディンバラ大学　医学研究評議会（MRC）　再生医学研究所（CRM）および産業クラスター訪問

＜スケジュール＞
7月24日　エディンバラ到着

7月25日　英国エディンバラ大学医学評議会（MRC）-再生医学研究所（CRM）と京都大学物質-細胞統合システム（iCeMS）合同学術シンポジウム参加

MRC-CRM視察

エディンバラ大学MRC-CRM関係者との懇談会

7月26日　iCeMS/京都SMI　スコットランド幹細胞企業合同シンポジウム参加・プレゼンテーション

Angel Biotechnology訪問

ロンドンへ移動

7月27日　AM　SC4SM訪問

PM　Altrika訪問・プレゼンテーション

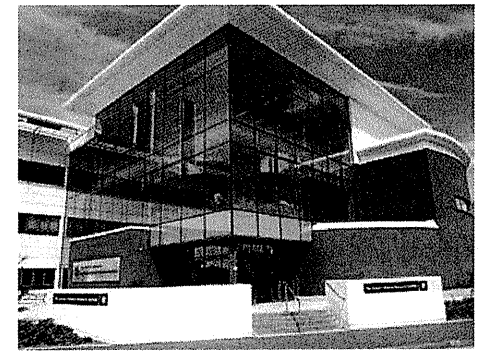
7月28日　帰国

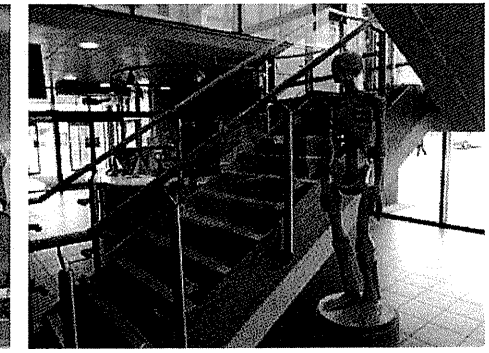
細胞育成士プロジェクトから
伊藤明良（M2）、藤岡瑠音(M1)の2名が参加
（京都大学大学院人間健康科学系専攻）

7月25日

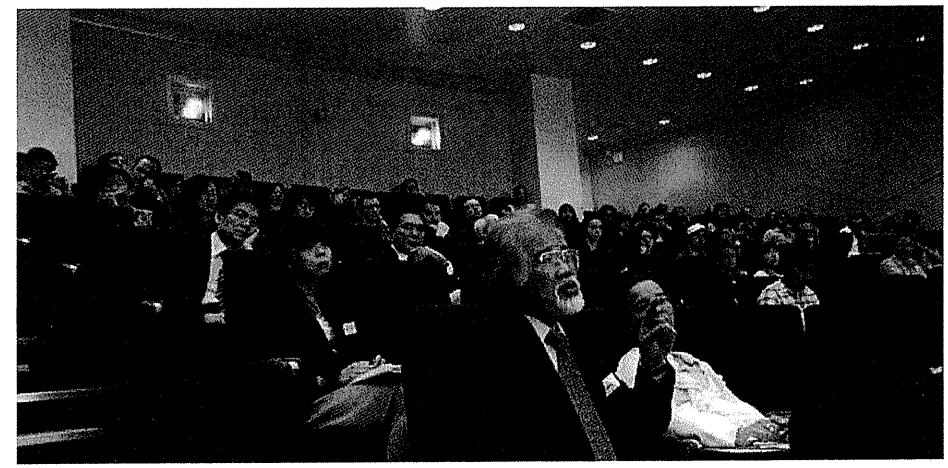
MRC-CRM　iCeMS合同シンポジウム

Next Generation Stem Cells: Tools and Technologies





会場：エディンバラ大学クイーンズ医学研究所（QMRI）

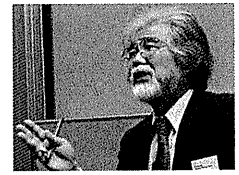


両機関の研究者10人が講演し、約150人が参加しました。
各講演では最新の研究成果とともに、多能性幹細胞（ES／iPS細胞など）の医学および産業応用に向けた技術や取り組みなどが発表されました。

＜スケジュール＞

9:00 **Prof. Charles ffrench-Constant**
Director's Introduction

9:15 **Prof. Norio Nakatsuji**
Multi-disciplinary research and application of pluripotent stem cells for disease mechanism research and drug discovery



9:45 **Dr. David Hay**
Generating Metabolically Active Hepatocytes from Pluripotent Stem Cells

10:15 **Prof. Motonari Uesugi**
Small Molecule Tools for Cell Biology and Cell Therapy

10:45 Comfort break

11:15 **Dr. Tilo Kunath**
Human iPS and ES cells to model neurodegenerative diseases

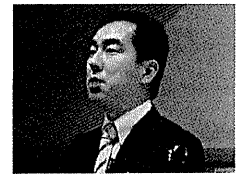
11:45 **Dr. Ludovic Vallier**
Genome editing in human induced pluripotent stem cells

12:15 Lunch break

13:15 **Dr. Shintaro Sengoku**
Study on innovation management for biomedical industrial clusters: Case comparison of Edinburgh and Kyoto

13:45 **Prof. Mark Bradley**
Polymer microarrays and the rapid identification of substrates for controlling stem cells

14 : 15 **Dr. Kenichiro Kamei**
Micro/Nano-engineered environments for controlling pluripotent stem cells



14 : 45 Comfort break

15 : 15 **Dr. Keisuke Kaji**
Reprogramming: From technology to biology

15 : 45 **Prof. Claus Nerlov**
Lineage specification in the hematopoietic system

Next Generation Stem Cells: Tools and Technologies Symposium

The MRC Centre for Regenerative Medicine
The University of Edinburgh

and the

Institute for Integrated Cell-Material Sciences
Kyoto University

Wellcome Auditorium
QMRI, Little France

Monday 25th July 2011
9.00 – 17.00

Entry is free but
YOU MUST REGISTER by contacting:
Fiona.Oswald@ed.ac.uk

Speakers

Prof Norio Nakatsuji
Prof Motonari Uesugi
Dr Kenichiro Kamei
Dr Shintaro Sengoku
Dr David Hay
Dr Keisuke Kaji
Dr Tilo Kunath
Prof Mark Bradley
Dr Ludovic Vallier
Prof Claus Nerlov

MRC Centre for Regenerative Medicine
The University of Edinburgh
Tel: 0131 242 6630

MRC Centre for Regenerative Medicine

THE UNIVERSITY of EDINBURGH

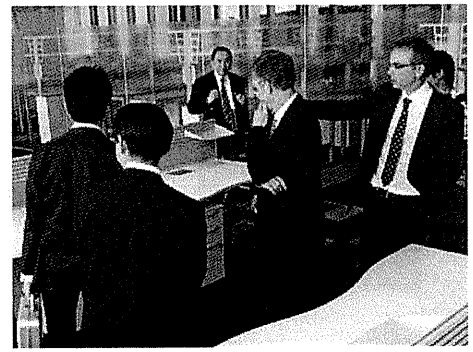
iCeMS

www.crm.ed.ac.uk/event/symposium-next-generation-stem-cells-tools-and-technologies

MRC-CRM視察

シンポジウム後は、総工費6千万ポンド（約76億円）のMRC-CRM新棟を視察する時間が設けられました。

エディンバラ大学MRC-CRM新棟外観



新棟の設備を紹介するゴードン・マクリーン
MRC-CRM最高執行責任者（右）

新棟の設備を紹介するフレンチコンスタント所長

バイオメディカル系ベンチャーを誘致するためのインキュベーション施設（奥）

エディンバラ大学MRC-CRM関係者との懇談会

クローン羊ドリーの研究で著名なイアン・ウィルムット卿（中央右）とも懇談でき、研究や人生のアドバイスを頂きました。

7月26日

iCeMS/京都SMI　スコットランド幹細胞企業合同シンポジウム

会場：エディンバラ大学chancellorビル

＜スケジュール＞

9：00　Welcome and introduction to EBQ and SDI by **Diane Harbison** and **Morag Nelson**.

Welcome and introduction to Stem Cell Research Project in Japan by **Takashi Asada**

9：30　Presentation by **Andy Carver**, Head of Business Development, Angel Biotechnology

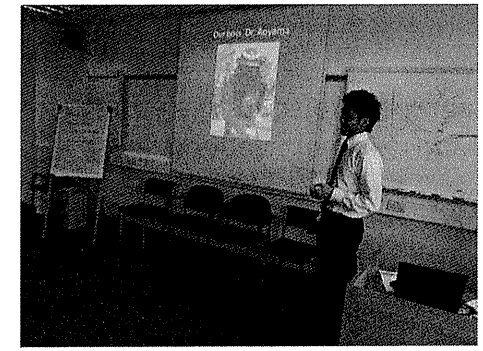
9：50　presentation by **Aidan Courtney**, CEO, Roslin Cells

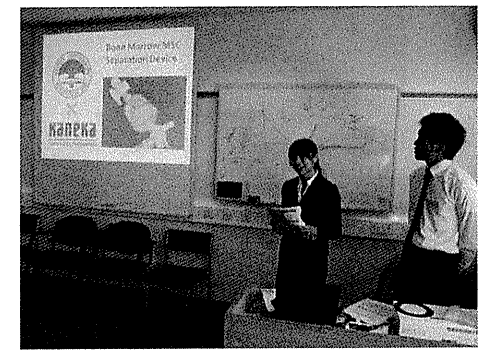
10:10　Refreshment break

10:30　Presentation by **Masaaki Takahashi**, Genetein

10:50　Presentation by **Verna McErlane**, Director of Commercial Operations, Sistemic

11:10　Presentation by **Rune Fujioka** and **Akira Ito**, Kaneka





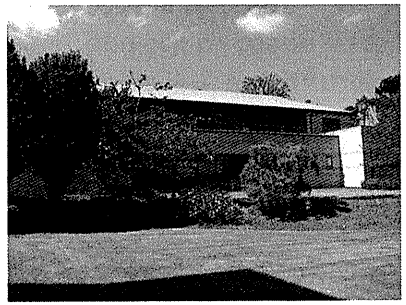
Kanekaと京都大学の共同研究により開発されたBone Marrow MSC Separation Deviceについて、約20分間発表しました。
多くの方に興味を持って頂き、デバイスの原理や実際的な使用方法などについて有意義な議論を行うことができました。

11:30 Presentation by **Kevin Price**, CEO, SSCN

11:50 Round table discussion

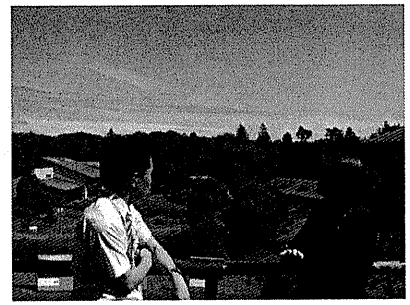
12:30 Networking Lunch

14:00 ～ **Angel Biotechnology訪問**



Angel Biotechnology

CCMT
(center for cell and mokecular therapy)



Angel Biotechnology関係者やスコットランド行政関係との懇談

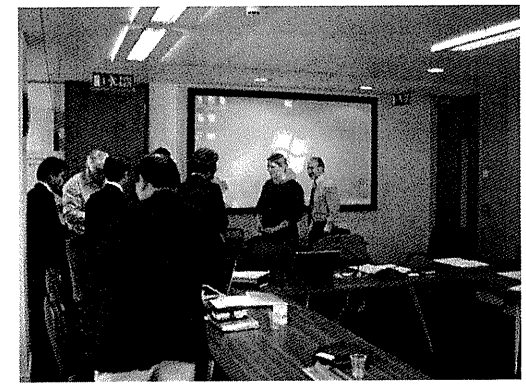
7月27日

SC4SM (Stem Cells for Safer Medicines)訪問

10:00 – 12:00 Discussion

Telephone conference to be prepared for
Japanese companies in Japan

Dr Frank Bonner, Chief Executive
of Stem Cells for Safer Medicines

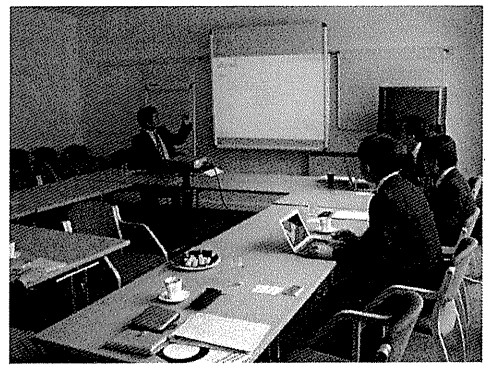


Altrika Ltd.訪問

15:30 – 17:30 Presentation by **Richard Snell**, Business Development Director, altrika

Presentation by **Kenichiro Kamei**, iCeMS

Presentation by **Akira Ito**, Kaneka

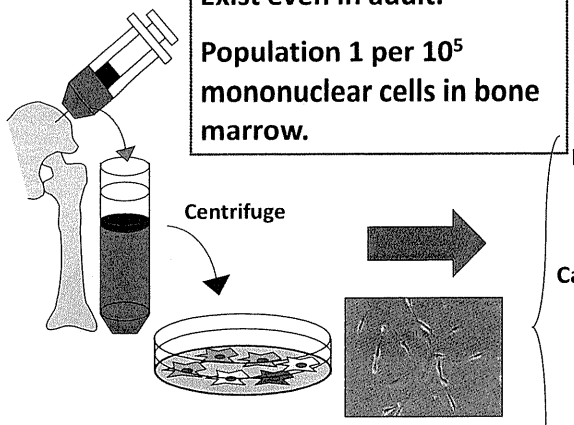


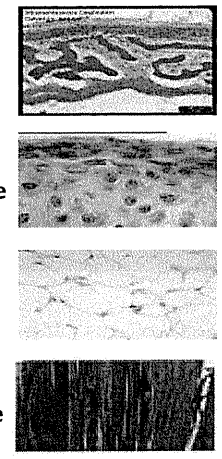
Bone Marrow MSC Separation Deviceについてプレゼンテーションを行いました。もともとaltrikaはこのデバイスに興味を持っていたこともあり、非常に興味を持って頂きました。

Mesenchymal Stem Cell (MSC)

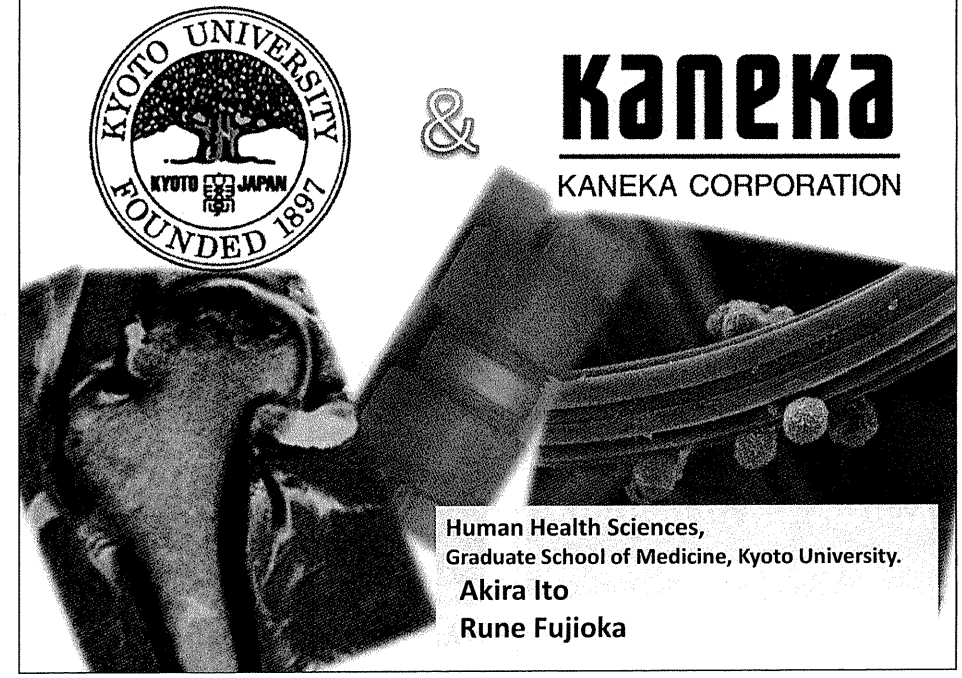
Exist even in adult.

Population 1 per 10^5 mononuclear cells in bone marrow.



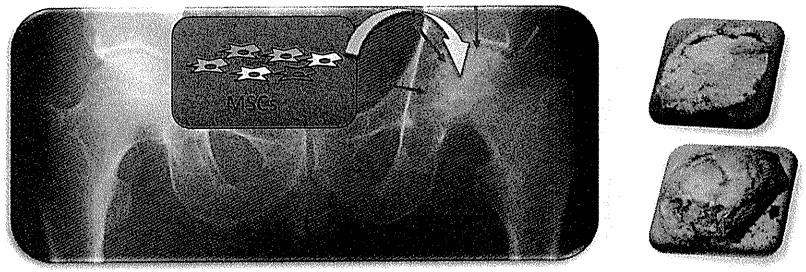


Plastic adherent fibroblastic cells with the potential to differentiate into multiple mesenchymal tissues.



Human Health Sciences,
Graduate School of Medicine, Kyoto University.
Akira Ito
Rune Fujioka

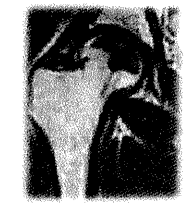
Osteonecrosis of femoral head



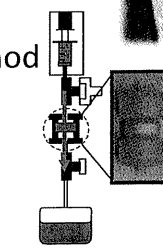
pathological condition	Etiology	Treatment
• Ischemia of femur head • Collapse of femur head by cell death	• Steroid • Alcohol • Unknown	• <u>No radical treatment</u> • Total hip replacement

Contents

- ✓ **Our clinical trial**
  - ➡ Mesenchymal stem cell(MSC)
  - ➡ Osteonecrosis of femoral head
  - ➡ MSC transplantation



- ✓ **New Device for isolating MSCs**
  - ➡ What the device is
  - ➡ Comparison with conventional method
  - ➡ Animal experiment



- ✓ **About us**

The issue for practical use

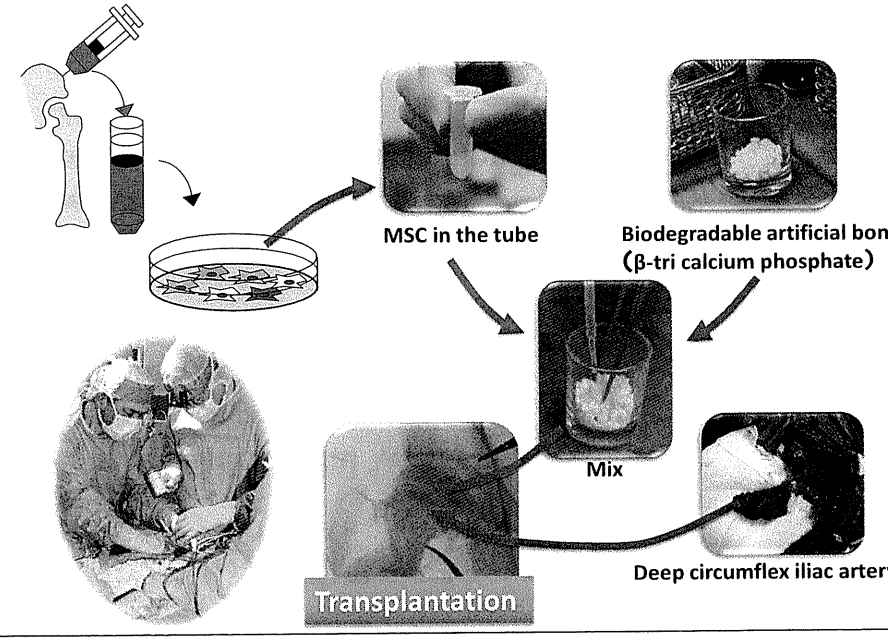
The cost of construction

We need a more efficient, handy, economical, and safer device.

⇒80 thousand pounds.

- Risk of contamination
- Time-consuming
- Operator's proficiency
- Too expensive

MSC transplantation (Phase Ⅰ - Ⅱ Clinical trial)



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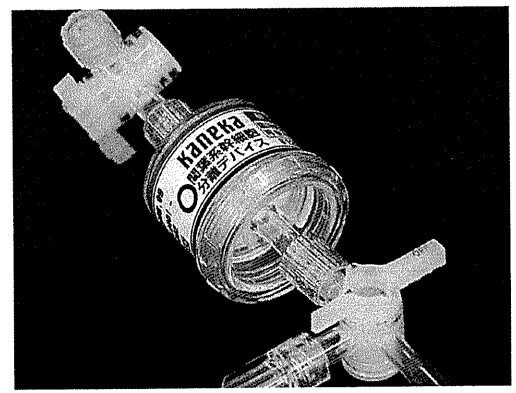
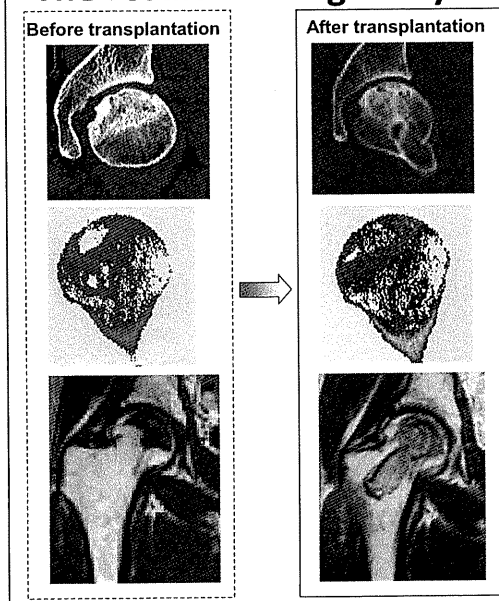
kaneka

KANEKA CORPORATION

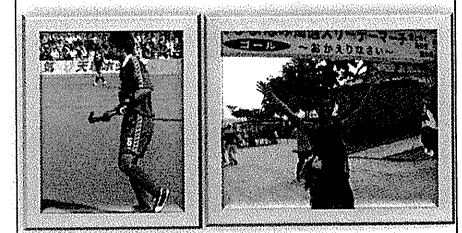
Bone Marrow MSC Separation Device



The result is being analyzed...

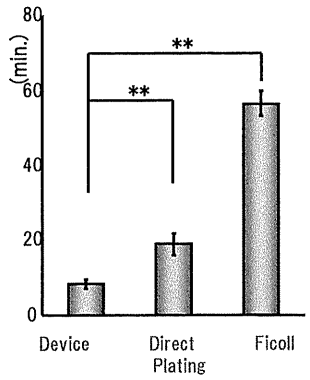


All the patient have returned to the society.



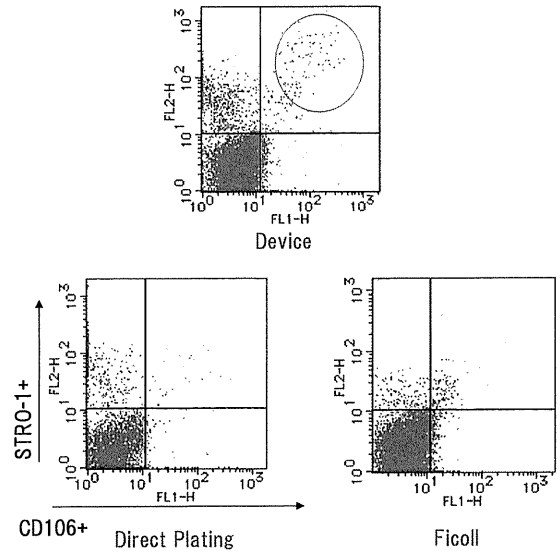
Number	Age	Number	Age
FB01	27	FB02	23
FB03	48	FB04	20
FB05	35	FB06	28
FB07	39	FB08	26
FB09	32	FB10	38

2. Processing Time

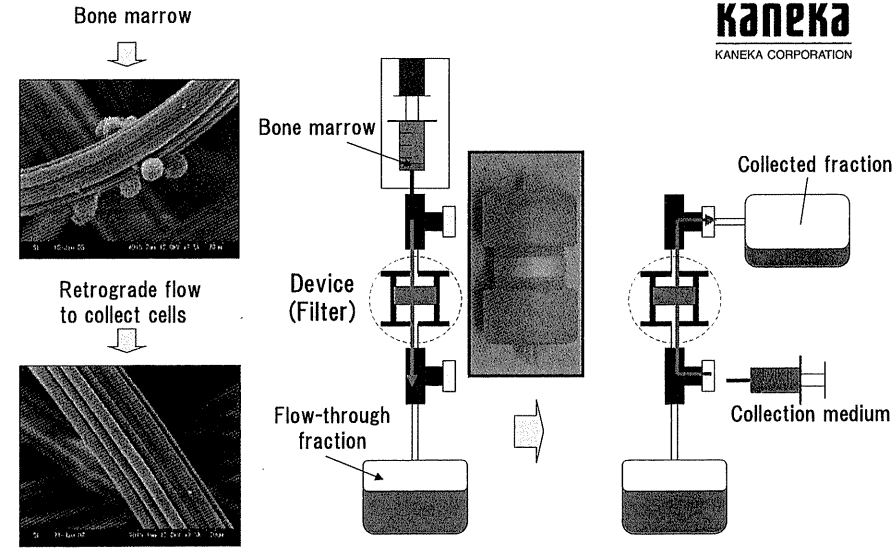


**; p<0.001

3. Expression of mesenchymal marker
Double positive population CD106+/STRO-1+

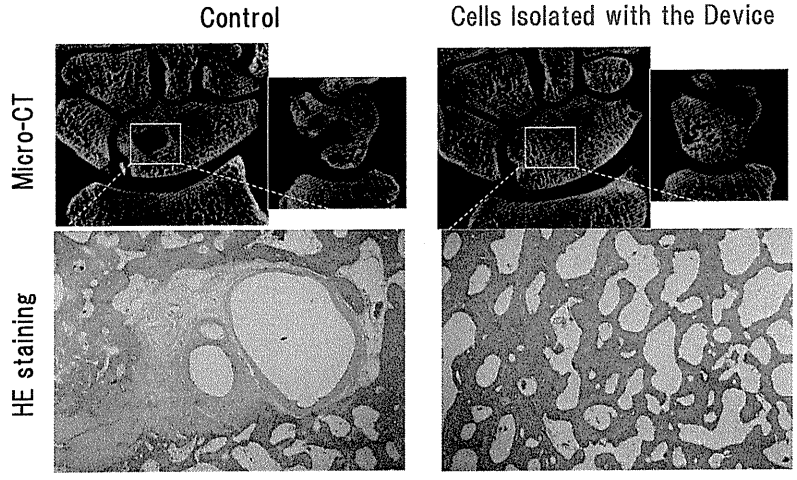


Development of New Device for isolating MSCs from bone marrow



Regeneration of Canine Bone Tissues

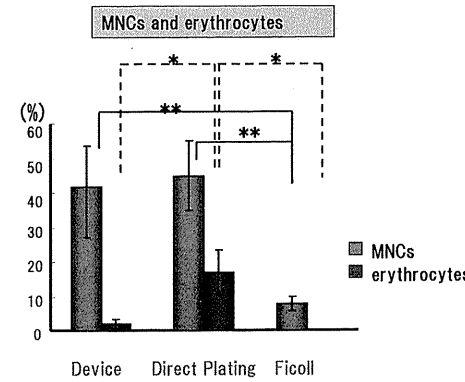
one year after the transplantation



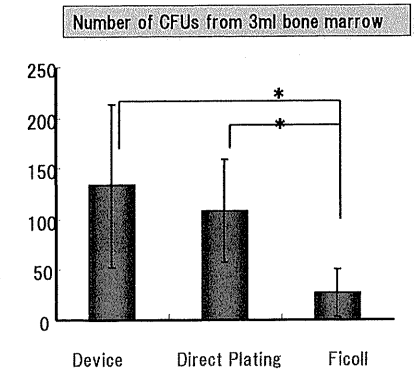
Efficacy & Safety ⇨ **Clinical use available**

Comparison of New Device with Conventional Method

1. Collection Efficacy



**; p<0.001, *; p<0.005



*; p<0.01

Bone Marrow MSC Separation Device

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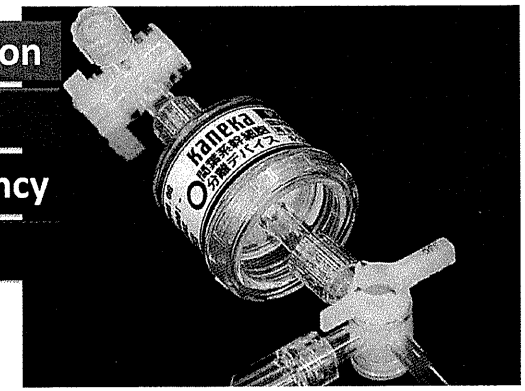
- Risk of contamination
- Time-consuming
- Operator's proficiency
- Too expensive

kaneka

KANEKA CORPORATION



研究成果の刊行に関する一覧表 （英文）

	発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
1	Yao H, Ashihara E, Strovel JW, Nakagawa Y, Kuroda J, Nagao R, Tanaka R, Yokota A, Takeuchi M, Sakai K, Shimazaki C, Taniwaki M, Strand K, Padia J, Hirai H, Kimura S, Maekawa T.	AV-65, a novel Wnt/β-catenin signal inhibitor, successfully suppresses progression of multiple myeloma in a mouse model.	Blood Cancer Journal	1(e43)	1-9	2011
2	KitawakiT, Kadowaki N , Fukunaga K, Kasai Y, Maekawa T, Ohmori K, Kondo T, Maekawa R, Takahara M, Nieda M, Kuzushima K, IshikawaT, Uchiyama T.	A phase I/IIa clinical trial of immunotherapy for elderly patients with acute myeloid leukaemia using dendritic cells co-pulsed with WT1 peptide and zoledronate .	Brit J Haematol	153	796-799	2011
3	Nagao R, Ashihara E, Kimura S, Strovel JW, Yao H, Takeuchi M, Tanaka R, Hayashi Y, Hirai H, Padia J, Strand K, Maekawa T.	Growth inhibition of imatinib-resistant CML cells with the T315I mutation and hypoxia-adaptation by AV65, a novel Wnt/β-catenin signaling inhibitor.	Cancer Lett	312	91-100	2011
4	Tanaka R, Kimura S, Ashihara E, Yoshimura M, Takahashi N, Wakita H, Itoh K, Nishiwaki K, Suzuki K, Nagao R, Yao H, Hayashi Y, Satake S, Hirai H, Sawada K-I, Ottmann OG, Melo JV, Maekawa, T.	Rapid automated detection of ABL kinase domain mutations in imatinib-resistant patients.	Cancer Lett	312	228-234	2011
5	Takeuchi M, Ashihara E, Maekawa T, et al.	Rakicidin A effectively induces apoptosis in hypoxia adapted Bcr-Abl positive leukemic cells.	Cancer Sci	102	591-596	2011
6	Kawatani M, Takayama H, Muroi M, Kimura S, Maekawa T, Osada H.	Identification of a small molecule inhibitor of DNA topoisomerase II by proteomic profiling.	Chem Biol	18	743-751	2011
7	Kitawaki T, Kadowaki N, Fukunaga K, Kasai Y, Maekawa T, Ohmori K, Itoh T, Shimizu A, Kuzushima K, Kondo T, Ishikawa T, Uchiyama T.	Cross-priming of CD8(+) T cells in vivo by dendritic cells pulsed with autologous apoptotic leukemic cells in immunotherapy for elderly patients with acute myeloid leukemia.	Exp Hematol	39	424-433	2011
8	Yao H, Ashihara E, Maekawa T.	Targeting the Wnt/β-catenin signaling pathway in human cancers.	Expert Opinion on Therapeutic Targets	15	873-887	2011
9	Kawata E, Ashihara E, Maekawa T.	RNA interference against Polo-like kinase-1 in advanced non-small cell lung cancers.	J Clin Bioinformatics	1	1-6	2011
10	Shoji T, Bando T, Fujinaga T, Chen F, Yurugi K, Maekawa T, Date H.	ABO-incompatible living-donor lobar lung transplantation.	J Heart Lung Transplant	30	479-480	2011

研究成果の刊行に関する一覧表 （英文）

	発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
11	Yamamichi J, Ojima T, Yurugi M, Iida M, Imamura T, Ashihara E, Kimura S, Maekawa T.	Single-step, label-free quantification of antibody in human serum for clinical applications based on localized surface plasmon resonance.	Nanomedicine: Nanotechnology, Biology, and Medicine	7	889-895	2011
12	Tauchi T, Okabe S, Ashihara E, Kimura S, Maekawa T, Ohayashiki K.	Combined effects of novel heat shock protein 90 inhibitor NVP-AUY922 and nilotinib in a random mutagenesis screen.	Oncogene	30	2789-2797	2011
13	Seno T, Hamaguchi M, Ashihara E, Kohno M, Ishino H, Yamamoto A, Kadoya M, Nakamura K, Murakami K, Matoba S, Maekawa T, Kawahito, Y.	15-Deoxy-Δ12,14 prostaglandin J2 reduces the formation of atherosclerotic lesions in apolipoprotein E knockout mice.	PLoS One	6: e25541	1-10	2011
14	Yamamoto-Sugitani M, Kuroda J, Ashihara E, Nagoshi H, Kobayashi T, Matsumoto Y, Sasaki N, Kiyota M, Nakayama R, Akaji K, Taki T, Uoshima N, Kobayashi Y, Horiike S, Maekawa T, Taniwaki M.	Galectin-3 induced by leukemia microenvironment promotes drug resistance and bone marrow lodgment in chronic myelogenous leukemia.	Proc Natl Acad Sci USA	108	17468-17473	2011
15	Tada N, Hinotsu S, Urushihara H, Kita F, Kai S, Takahashi T, A, Kato S, Takanashi M, Ito K, Sawai H, Maekawa, T, Kosugi S, Kawakami K.	The current status of umbilical cord blood collection in Japanese medical centers: survey of obstetricians.	Transfusion and Apheresis Science	44	263-268	2011
16	Maita S, Yuasa T, Tsuchiya N, Mitobe Y, Narita S, Horikawa Y, Hatake K, Fukui I, Kimura S, Maekawa T, Habuchi T.	Antitumor effect of sunitinib against skeletal metastatic renal cell carcinoma through inhibition of osteoclast function. Int J Cancer,	Int J Cancer	130	677-684	2012
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ORIGINAL ARTICLE

AV-65, a novel Wnt/β-catenin signal inhibitor, successfully suppresses progression of multiple myeloma in a mouse model

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Multiple myeloma (MM) is a malignant neoplasm of plasma cells. Although new molecular targeting agents against MM have been developed based on the better understanding of the underlying pathogenesis, MM still remains an incurable disease. We previously demonstrated that β-catenin, a downstream effector in the Wnt pathway, is a potential target in MM using RNA interference in an *in vivo* experimental mouse model. In this study, we have screened a library of more than 100 000 small-molecule chemical compounds for novel Wnt/β-catenin signaling inhibitors using a high-throughput transcriptional screening technology. We identified AV-65, which diminished β-catenin protein levels and T-cell factor transcriptional activity. AV-65 then decreased c-myc, cyclin D1 and survivin expression, resulting in the inhibition of MM cell proliferation through the apoptotic pathway. AV-65 treatment prolonged the survival of MM-bearing mice. These findings indicate that this compound represents a novel and attractive therapeutic agent against MM. This study also illustrates the potential of high-throughput transcriptional screening to identify candidates for anticancer drug discovery.
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Keywords: multiple myeloma; Wnt; β-catenin; high-throughput screening

Introduction

Multiple myeloma (MM) is a malignant neoplasm of plasma cells characterized by frequent chromosomal translocations and gene mutations.[1] High-dose chemotherapeutics in conjunction with hematopoietic stem cell transplantation has been one standard clinical course of the treatment of MM.[2–4] As we gain a better understanding of the pathogenesis underlying MM, new molecular targeting agents can be developed.[5,6] Nevertheless, MM remains incurable at present.[7] Thus, it is important to continue to investigate new therapeutic agents that focus on targeting the biology of MM cells.

β-Catenin is a downstream effector in the canonical Wnt signaling pathway; thus, it is involved in the regulation of cell fate, proliferation and the self-renewal of stem cells. In the absence of Wnt ligands, cytosolic β-catenin undergoes proteasomal degradation through sequential phosphorylation and ubiquitination.[8,9] In the process, β-catenin that is phosphorylated by casein kinase I, and subsequently glycogen synthase kinase-3β, forms a degradation complex comprised of glycogen synthase kinase-3β, casein kinase I, axin and adenomatous polyposis coli. Phosphorylated β-catenin is ubiquitinated by cellular β-transducin repeat-containing proteins (β-TrCP), and subsequently degraded by the 26S proteasome.[10] Upon Wnt ligand binding to Frizzled receptors and the co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6, the dishevelled phosphoprotein inactivates glycogen synthase kinase-3β, and the β-catenin degradation complex dissociates, resulting in the stabilization of β-catenin. Stabilized β-catenin translocates to the nucleus, where it binds to lymphoid enhancer-binding factor (LEF) and T-cell factor (TCF) proteins to activate the transcription of target genes such as *c-myc* and *cyclin D1*, which are involved in cell proliferation and survival.[11,12] Activation of Wnt signaling is closely linked to the process of carcinogenesis.[13] Human MM-derived cell lines and primary MM cells overexpress β-catenin, whereas in normal plasma cells, β-catenin expression is undetectable.[14–16] The recent study demonstrated that genes of soluble Wnt signal antagonists were methylated in MM cells, resulting in repression of their transcripts, and consequently, the increased expression of β-catenin.[17] Overexpression of β-catenin promotes transcriptional activity of target genes related to the proliferation of MM cells, whereas inhibition of Wnt/β-catenin signaling suppresses MM growth.[15,16] These results suggest that β-catenin might be an attractive therapeutic target for MM.

Progress in molecular biology has enabled significant advances in drug discovery strategies. For example, RNA interference is a technique that can be used to knockdown the expression of specific genes in treated cells. An altered transcriptional profile can then be used as a biomarker for a specific molecular targeting, and quantitative evaluation can be applied to high-throughput technology.[18,19] Using high-throughput transcriptional screening technology (Supplementary Figure 1), we identified effective inhibitors of the Wnt/β-catenin signaling from a library of more than 100 000 chemical compounds (Supplementary Figure 2). From this initial series, we optimized a novel Wnt/β-catenin signal inhibitor, AV-65, that significantly suppressed the growth of MM cells and prolonged survival in a mouse model of orthotopic MM. These results demonstrate that AV-65 is a novel and attractive therapeutic agent for the treatment of MM.

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Materials and methods

Cell lines, reagents and animals

Human AMO-1, KMS-12-BM, RPMI8226, NCI-H929, U226 and OPM-2 myeloma cell lines, and the IM-9 Epstein–Barr virus-transformed cell line derived from an MM patient, were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). RPMI8226/LR5 melphalan-resistant cells were kindly provided by Dr William S Dalton (University of South Florida, Tampa, FL, USA).[20] HCT-15 colorectal adenocarcinoma cells were purchased from American Tissue Culture Collection (Manassas, VA, USA). The 293T cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. IM-9, RPMI8226, NCI-H929 and U266 cells were cultured in RPMI1640 (Gibco, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Tokyo, Japan), 2 mM L-glutamine (Gibco) and 1% penicillin–streptomycin (Gibco). The AMO-1 and KMS-12-BM cell lines were cultured in RPMI1640 containing 20% FCS, 2 mM L-glutamine and 1% penicillin–streptomycin. The RPMI8226/LR5 cell line was cultured in media containing 10% FCS, 2 mM L-glutamine, 1% penicillin–streptomycin and 5 μM melphalan (Sigma-Aldrich, Tokyo, Japan). Hepatocytes were cultured using CS-C complete medium kit (Cell Systems, Kirkland, WA, USA). HCT-15 cells were cultured in RPMI1640 containing 5% FCS, 2 mM L-glutamine and 1% penicillin–streptomycin. The 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin–streptomycin. All cell lines were maintained at 37 °C in a fully humidified atmosphere of 5% CO_2 in air.

Peripheral blood mononuclear cells from three healthy volunteers were isolated by using Ficoll–Hypaque (GE Healthcare Japan, Tokyo, Japan), and cultured with in Iscove's modified Dulbecco's medium (Gibco) containing 20% FCS, 2 mM L-glutamine and 1% penicillin–streptomycin. Bone marrow (BM) samples were obtained from MM patients who were hospitalized in the Hospital of Kyoto Prefectural University of Medicine. In accordance with the Declaration of Helsinki recommendations, all procedures were approved by the institutional review board at Kyoto Prefectural University of Medicine, and written informed consent was obtained from every participant. Ficoll–Hypaque density centrifugation was used to separate mononuclear cells, followed by magnetic cell sorting (Miltenyi, Gladbach, Germany) using an anti-CD138 antibody (Ab) (Miltenyi) to enrich BM-derived MM cells. Cells were analyzed by FACS Canto II using the Diva software (BD Bioscience, Tokyo, Japan). The purity of the enriched CD138-positive cell populations was $\geqslant$95%. Isolated MM cells were cultured in RPMI1640 containing 10% FCS, 2 mM L-glutamine and 20 ng/ml human interleukin-6 (R&D Systems, Minneapolis, MN, USA).

Specific pathogen-free 6- to 8-week old female nonobese diabetic-severe combined immune deficiency (NOD/SCID) mice (Clea Japan, Tokyo, Japan) were used for the *in vivo* experiments. Approval for these studies was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

Growth inhibitory effects on myeloma cells

Cell proliferation was evaluated by the modified MTT (3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay using Cell-Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan), as described previously.[21,22] Cells were seeded in a flat-bottomed 96-well plate (BD Bioscience) at a density of 3×10^3 cells in 100 μl of medium per well, and then incubated with serial dilutions of AV-65 for 72 h. The mean of four samples at each concentration was evaluated. Half maximal inhibitory concentration values were obtained using the nonlinear regression program CalcuSyn (Biosoft, Cambridge, UK).

Western blot analysis

Following treatment with AV-65, more than 1×10^6 cells were collected by centrifugation, and then the cells were washed with ice-cold phosphate-buffered saline (−) twice. Ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 5 mM EDTA, 20 mM NaF, 1% NP-40) containing fresh phenylmethylsulfonyl (1 mM) and protease inhibitor (10 μg/ml) was added to the cells. The suspension was transferred into a centrifuge tube and placed on ice for 15 min (min) with occasional vortexing to ensure complete lysis of the cells. The cell suspension was cleared by centrifugation at 14 000 *g* for 30 min at 4 °C. Nuclear and cytoplasmic protein fractions were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. The supernatants (total cell lysate, nuclear and cytoplasmic protein fractions) were either used immediately or stored at −80 °C. Protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories, Osaka, Japan). Immunoblotting was performed as described previously.[16,21] Samples (20 μg of protein) were analyzed using the following primary Abs, as indicated: anti-β-catenin (BD Pharmingen, San Jose, CA, USA), -Bad (Stressgen, Victoria, BC, Canada), -Bid (a kind gift from Dr David CS Huang, The Walter and Eliza Hall Institute of Medical Research (WEHI), Parkville, VIC, Australia),[23] -Bim (clone 3C5, produced by Dr LA O'Reilly (WEHI)), -Bcl-2 (Bcl-2-100; Upstate, Lake Placid, NY, USA), -Bcl-xL (Stressgen), -Puma (ProSci, Poway, CA, USA), -Noxa (Alexis Biochemicals, Lausen, Switzerland), -Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), -c-myc (Santa Cruz Biotechnology), -cyclin D1 (BD Pharmingen), -Oct-1 (Santa Cruz Biotechnology,), survivin (Cell Signaling Technology, Danvers, MA, USA) and -actin (Sigma-Aldrich). Horseradish peroxidase-coupled immunoglobulin G (Amersham Biosciences, Tokyo, Japan) was used as a secondary Ab, and immunoreactive proteins were detected by enhanced chemiluminescence or ECL-plus kits (Amersham Biosciences).

Ubiquitination of β-catenin

At 12 h after AV-65 treatment, whole-cell lysates were obtained as described above. Lysates were subjected to immunoprecipitation using an anti-β-catenin monoclonal Ab (BD Pharmingen) and Dynabeads Protein A (Invitrogen), according to the manufacturer's instructions. Ubiquitination of β-catenin was detected with anti-mono- and anti-poly-ubiquitinyl conjugates (Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA).

TCF/LEF dual luciferase reporter assay

The activity of TCF/LEF transcription in HCT-15 cells was evaluated with the Wnt Cignal Reporter Assay (SABioscience, Fredrick, MD, USA). HCT-15 colorectal cancer cell line expresses high levels of β-catenin[24] and is easily transfectable with plasmids. For each sample, 3×10^4 HCT-15 cells were reverse-transfected with 100 ng of a TCF/LEF firefly luciferase reporter plasmid and a constitutively expressing CMV-driven

Renilla luciferase reporter with SureFECT Transfection Reagent (SABioscience, Fredrick, MD, USA), according to the manufacturer's instructions. At 16 h post-transfection, media were changed to assay media (Opti-MEM containing 0.5% FBS and 1% non-essential amino acids) for 8 h, followed by AV-65 treatment for 14 h. Relative luciferase activity of cells was detected using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and a Wallac Victor 1420 Plate Reader (Perkin-Elmer, Waltham, MA, USA). The relative luciferase activities generated by each well were calculated by determining the ratio of the luciferase (firefly/*Renilla*) values to normalize the signal. Three independent transfections were carried out in duplicate for each of the concentrations tested.

Induction of apoptosis and cell cycle analysis

Cell cycle analysis using propidium iodide (PI) was performed as described previously.[22] Apoptosis induced by AV-65 was determined using Annexin-V-FITC Apoptosis Detection Kit I (BD Bioscience), according to the manufacturer's instructions. Cells were analyzed by FACS Canto II using the Diva software (BD Bioscience). Caspase-3, -8 and -9 activities in the presence of AV-65 were evaluated using a fluorometric protease assay kit (MBL, Aichi, Japan), according to the manufacturer's instructions.

Lentiviral transduction

We produced β-TrCP knockdown IM-9 cells using a lentivirus vector expressing short hairpin RNA against β-TrCP. Plasmids expressing short hairpin RNA against β-TrCP and green fluorescent protein were provided by Dr Kenzo Tokunaga (International Medical Center of Japan, Tokyo, Japan). The lentiviral packaging plasmid psPAX2 was provided by Addgene (Addgene plasmid 12260, D Trono, Lausanne, Switzerland). The pHIT/G plasmid[25] expressing the vesicular stomatitis virus-G protein was kindly provided by M Malim (University of Pennsylvania, Philadelphia, PA, USA). In all, 10 μg of β-TrCP short hairpin RNA, 10 μg of psPAX2 and 10 μg of pHIT/G were added to a mixture of BES (*N,N*-bis[2-hydroxy-ethyl]-2-aminoethanesulfonic acid)-buffered saline and calcium chloride. After incubation for 20 min, the solution was added dropwise to a 10 cm dish of 70% confluent 293T cells. The cells were incubated for 12 h at 37 °C in a humidified incubator with an atmosphere of 3% CO_2. The medium was replaced with 10 μM forskolin-containing medium and incubated at 37 °C and 10% CO_2. After 48 h, the supernatants were harvested. In total, 1.0×10^6 IM-9 cells were transduced with 3 ml of the supernatant and polybrene (Sigma-Aldrich). We isolated GFP-positive cells by using a limiting dilution method.

Real-time polymerase chain reaction

RNA was purified with the QIAamp RNA Blood Mini Kit (Qiagen, Tokyo, Japan) and subjected to reverse transcription. Human *β-TrCP* gene expression levels were measured by a real-time polymerase chain reaction. A real-time polymerase chain reaction mixture contained 4 μl of Taqman master mix (Roche Diagnostics GmbH, Mannheim, Germany), cDNA, pairs of primers and Taqman probe (Universal Probe Library; Roche Diagnostics GmbH). The cDNA was amplified with a Light Cycler 3.5 (Roche Diagnostics GmbH) using the following parameters: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Glyceraldehyde-3-phosphate was used as an internal to normalize the loading difference. The primers for β-TrCP were as follows: forward primer, CCAGATAAATAA CCATACACTGACCTC and reverse primer, CAGATACGTTAAA TACCGCAACTTT. The primers for glyceraldehyde-3-phosphate were as follows: forward primer, AGCCACATCGCTCAGACAC and reverse primer, GCCCAATACGACCAAATCC.

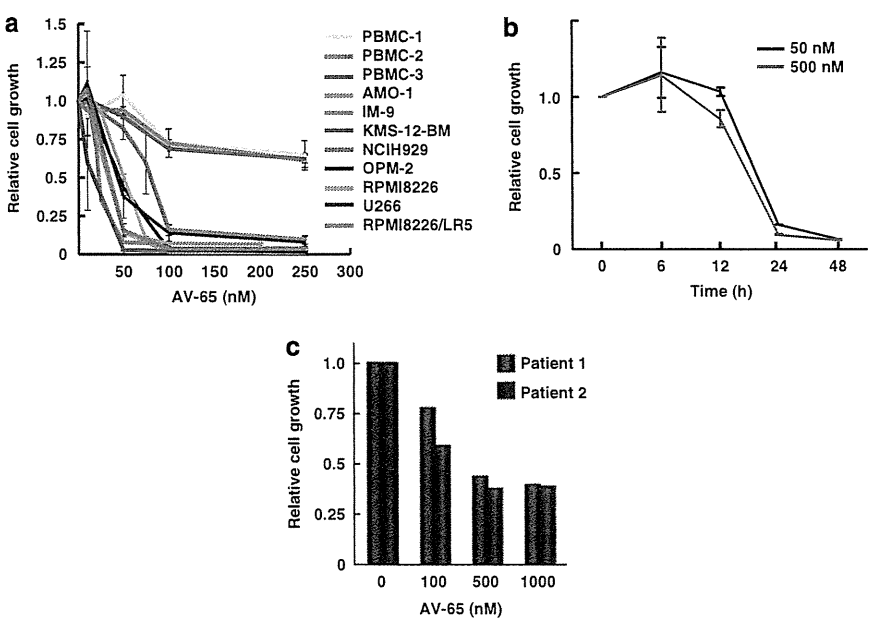

Figure 1 Inhibition of myeloma cell proliferation. (**a**) The dose-dependent effect of compounds on the proliferation of myeloma cell lines was evaluated by the modified MTT assay. Cells were incubated with serial dilutions of compounds for 72 h. Data represent the means ± s.d. of three independent experiments, and four replicate experiments. (**b**) Time-dependent inhibition of proliferation of IM-9 cells. Cells were incubated with AV-65 for 6, 12, 24 and 48 h. Data represent the means ± s.d. of three independent experiments, and four replicate experiments. (**c**) Effect of compounds on CD138-positive myeloma cells and CD138-positive normal plasma cells. CD138-positive myeloma cells and CD138-normal plasma cells were purified from BM mononuclear cells obtained from myeloma patients and healthy volunteers, respectively. Data represent the mean of four replicates for each concentration.