body weight was reversed by the anti-SHPS-1 mAb treatment. The administration of 10 or 100 μg anti-SHPS-1 mAb recovered the body weight almost completely to the level of non-CIA mice. The increase of body weights in those 2 groups was significantly greater than that of the control IgG group (both at $p < 0.05$). Although the development of arthritis was strongly inhibited by MTX, the mice treated with MTX lost approximately 2.9 g in body weight during the experimental period, which might be ascribed to the toxic effects of the immunosuppressant.

In the control mice, the clinical score started to increase on Day 23 and reached a maximum on Day 31 (Figure 1C). The administration of 10 or 100 μg anti-SHPS-1 mAb significantly improved the clinical score on Day 31 and later. Since improvement was not observed in mice treated with 1 μg anti-SHPS-1 mAb, the critical dose of antibody treatment for the mice was considered to be between 1 and 10 μg per injection. Interestingly, the improvement in clinical score was maintained until Day 41, 10 days after the last antibody administration. In mice treated with MTX, the development of arthritis was completely inhibited.

Consistent results were obtained by the measurement of footpad thickness (Figure 1D). In control mice, the footpad thickness started to increase on Day 25, and it continued to increase until Day 35, while no increase was observed in the MTX-treated mice. In mice treated with 10 or 100 μg anti-SHPS-1 mAb, the increase of footpad thickness was inhibited on Day 27 and later. In accord with the clinical score, 1 μg anti-SHPS-1 mAb was not enough to show the effect.

Anti-SHPS-1 antibody ameliorated the severity of established arthritis. We then investigated whether the administration of anti-SHPS-1 mAb could reduce the severity of established arthritis. In this experiment, the antibody treatment was commenced after the onset of arthritis (on Day 29), and the severity of arthritis was evaluated by the clinical score and footpad thickness of the hind limbs. The clinical score was reduced significantly as early as 2 days after the first injection of 100 μg anti-SHPS-1 mAb (Figure 2A). The reduction in clinical score became more obvious, and this was maintained until Day 45, 6 days after the completion of antibody administration.

In accord with the change of clinical score, the antibody treatment reduced the footpad thickness at 2 days and later after the initiation of antibody treatment (Figure 2B). Similar to the clinical score, the reduction in footpad thickness was maintained until Day 45.

Histological evaluation. Next, the effect of the antibody treatment was evaluated by histology. The mice were given 6 injections of 10 or 100 μg anti-SHPS-1 mAb after the onset of arthritis following Protocol B, and sacrificed 6 days after the last antibody administration. H&E-stained sections of ankle and tarsal joints were prepared, and the severity of arthritic change was evaluated by scores that were compared with those of mice treated with control IgG.

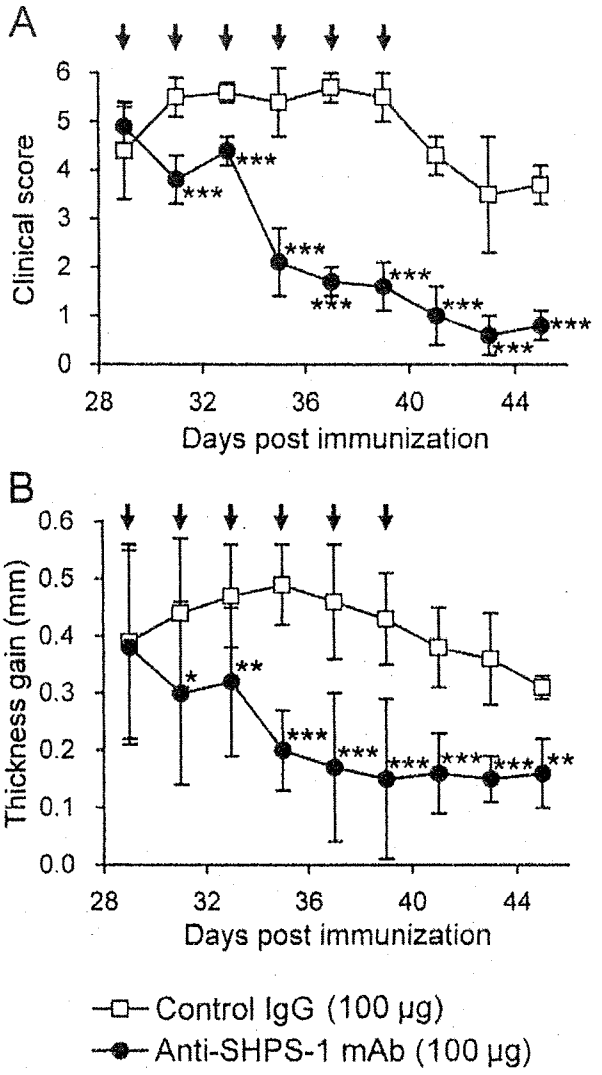

Figure 2. Effect of anti-SHPS-1 mAb treatment on severity of established arthritis. 100 μg of anti-SHPS-1 mAb or control IgG was given to mice after onset of arthritis, and severity of arthritis was evaluated by clinical score (A) and gain of footpad thickness (B), from the beginning of the treatment until 2 weeks after its end. Black arrows indicate the timing of antibody administration. Data are mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control IgG.

In control mice, severe arthritic change with obvious inflammatory cell infiltration and bone erosion was observed within and around the ankle and tarsal joints (Figure 3A-3C). Although the anti-SHPS-1 antibody was given after the onset of arthritis, the severity of arthritic change was considerably reduced in mice treated with anti-SHPS-1 mAb (Figure 3D-3F). Thus, the scores for inflammatory cell infiltration and those for bone destruction were significantly reduced in the antibody-treated mice (Figure 3G and 3H, respectively).

Anti-SHPS-1 antibody did not affect induction of anti-type II collagen antibodies. In CIA mice, arthritis is caused by

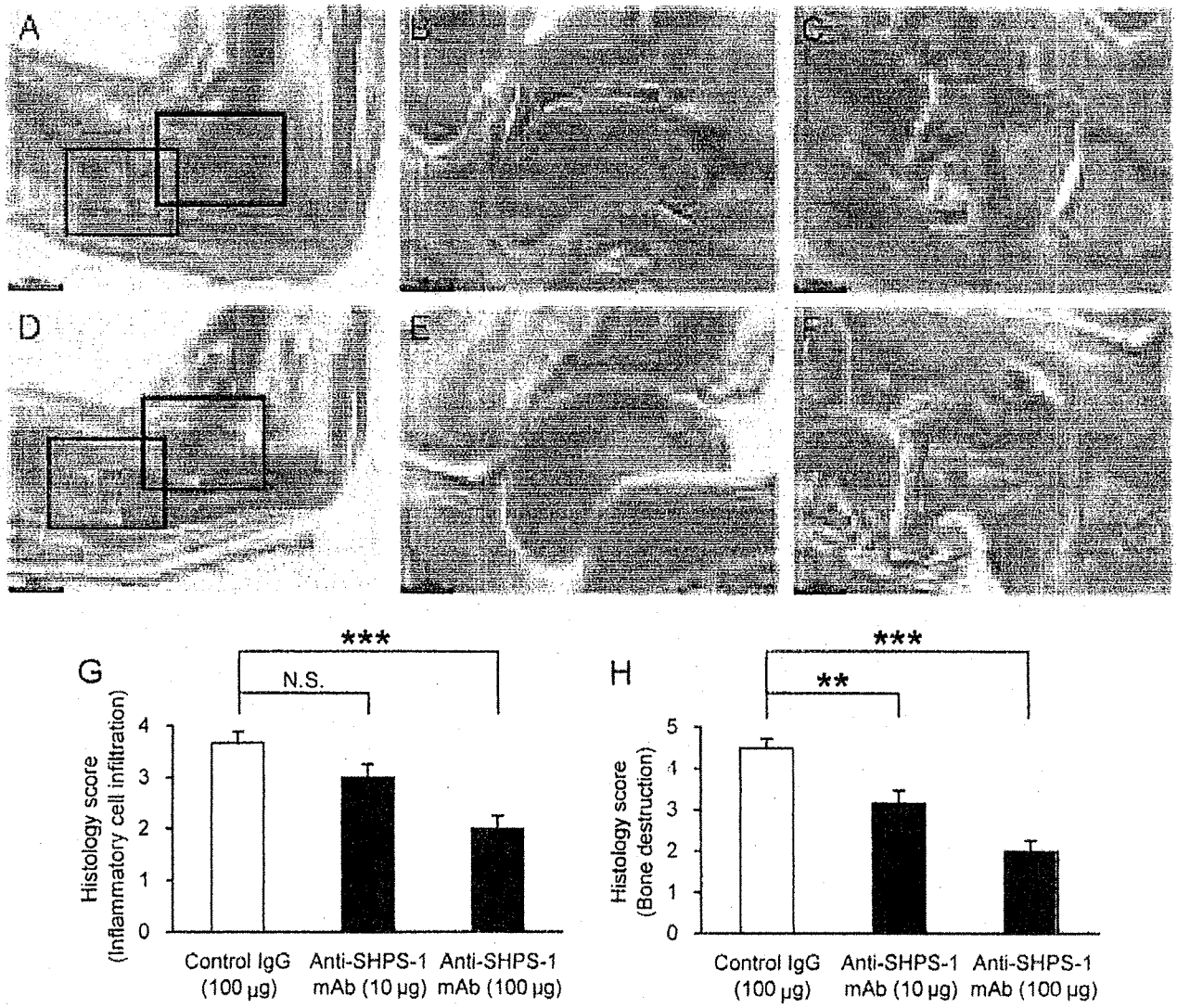

Figure 3. Histological evaluation of ankle and tarsal joints in antibody-treated and control mice. (A-C) In control mice given 100 μg control IgG, obvious synovial thickening and pannus formation was observed, together with marked inflammatory cell infiltration. Bone erosion occurred at multiple sites, which often extended deep into the subchondral bone. (D-F) In mice treated with 100 μg anti-SHPS-1 mAb, severity of synovial thickening and extent of inflammatory cell infiltration were considerably reduced. Area of bone erosion was also reduced, and rarely extended into the subchondral bone. Higher magnification images of inset areas in A and D are shown in B and C, and E and F, respectively. Scale bars are 1000 μm in A and D, and 300 μm in B, C, E, and F. H&E staining. (G and H) Histological scores for inflammatory cell infiltration (G) and bone destruction (H) in mice treated with 10 or 100 μg anti-SHPS-1 mAb are shown together with those for mice given 100 μg control IgG. Data are mean ± SD. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with control IgG.

autoimmune mechanisms that involve both humoral and cellular immune responses to CII[30,31]. Thus, we next determined the effect of the anti-SHPS-1 antibody treatment on the humoral response by measuring the concentration of anti-CII antibodies in sera. In this experiment, administration of anti-SHPS-1 mAb was started on the day of the second immunization, following Protocol A, and blood was obtained 2 weeks after the end of treatment. Comparison of the results between the antibody-treated mice and those of mice given control IgG or MTX revealed that the induction of anti-CII antibodies was not affected by the antibody treatment. This implies that the therapeutic effect of anti-SHPS-1 mAb is likely through the suppression of T cell responses, rather than via the modulation of B cell function.

Anti-SHPS-1 antibody and anti-CD47 antibody inhibited osteoclast formation. The observation that bone destruction was significantly reduced by the administration of anti-SHPS-1 mAb led us to hypothesize that the antibody could inhibit osteoclast formation. We then tested this hypothesis by an *in vitro* experiment. We also examined the effect of anti-CD47 mAb on osteoclast formation. In this experiment, murine bone marrow cells were obtained and osteoclast formation was induced in the presence of anti-SHPS-1 mAb or anti-CD47 mAb. The results clearly indicated that those

antibodies both inhibited the formation of osteoclasts in a dose-dependent manner (Figure 4). The inhibition was more obvious with anti-SHPS-1 mAb. With this antibody, the number of osteoclasts was significantly reduced with as little as 0.05 μg/ml of the antibody, and osteoclast formation was almost completely abrogated at the concentration of 2.5 μg/ml. Compared with this, the effect of anti-CD47 mAb was considerably lower. The ratio of inhibition did not reach 50% even with 10 μg/ml of this antibody.

Anti-SHPS-1 antibody and anti-CD47 antibody reduced secretion of proinflammatory cytokines from ConA-stimulated murine spleen cells. In order to determine the effects of anti-SHPS-1 mAb and anti-CD47 mAb on cytokine release from lymphatic cells, murine spleen cells were stimulated with ConA in the presence of anti-SHPS-1 mAb or anti-CD47 mAb, and cytokine concentrations in the media were determined. Upon stimulation with ConA, the spleen cells released all measured pro- and antiinflammatory cytokines to the media. The effect of anti-SHPS-1 mAb on cytokine release differed among the cytokines (Figure 5A-5G). The release of IL-1ß, IL-2, IL-12, IFN-γ, and TNF-α into the media was suppressed by the antibody, while that of IL-4 or IL-10 was almost unaffected. The suppression was most obvious for IFN-γ, with as little as 0.02 μg/ml of the antibody significantly reducing its release. The IC_{50} values of IL-1ß, IL-2, IL-12, IFN-γ, and TNF-α were 0.68, 0.50, 0.16,

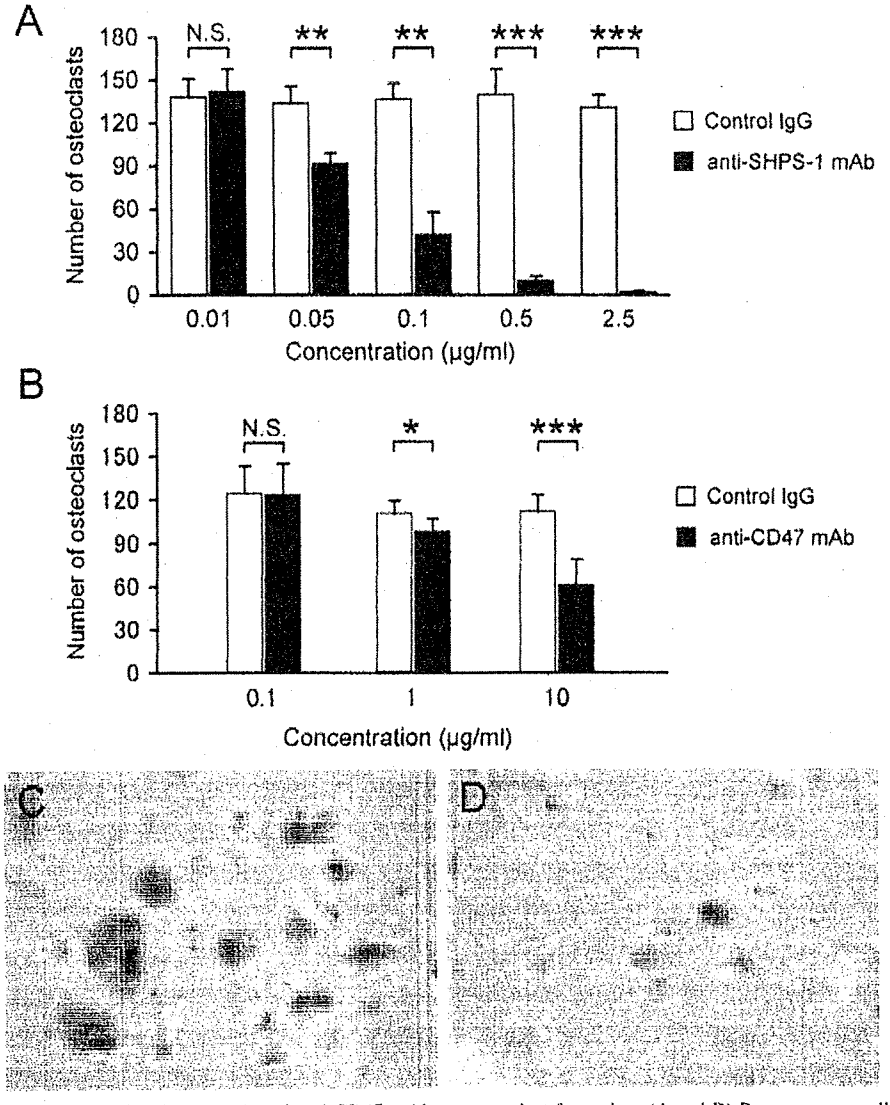

Figure 4. Effect of anti-SHPS-1 mAb and anti-CD47 mAb on osteoclast formation. (A and B) Bone marrow cells were obtained from Balb/c mice, and formation of osteoclasts was induced by M-CSF and RANKL for 5 days, in the presence of various concentrations of anti-SHPS-1 mAb or control IgG (A), and anti-CD47 mAb or control IgG (B). Number of TRAP-positive multinucleated cells in each well is shown. Data are mean ± SD. **p < 0.01 and ***p < 0.001 against control IgG. (C and D) Formation of osteoclasts in the presence of control IgG (2.5 μg/ml; panel C) or anti-SHPS-1 mAb (2.5 μg/ml; panel D). TRAP staining.

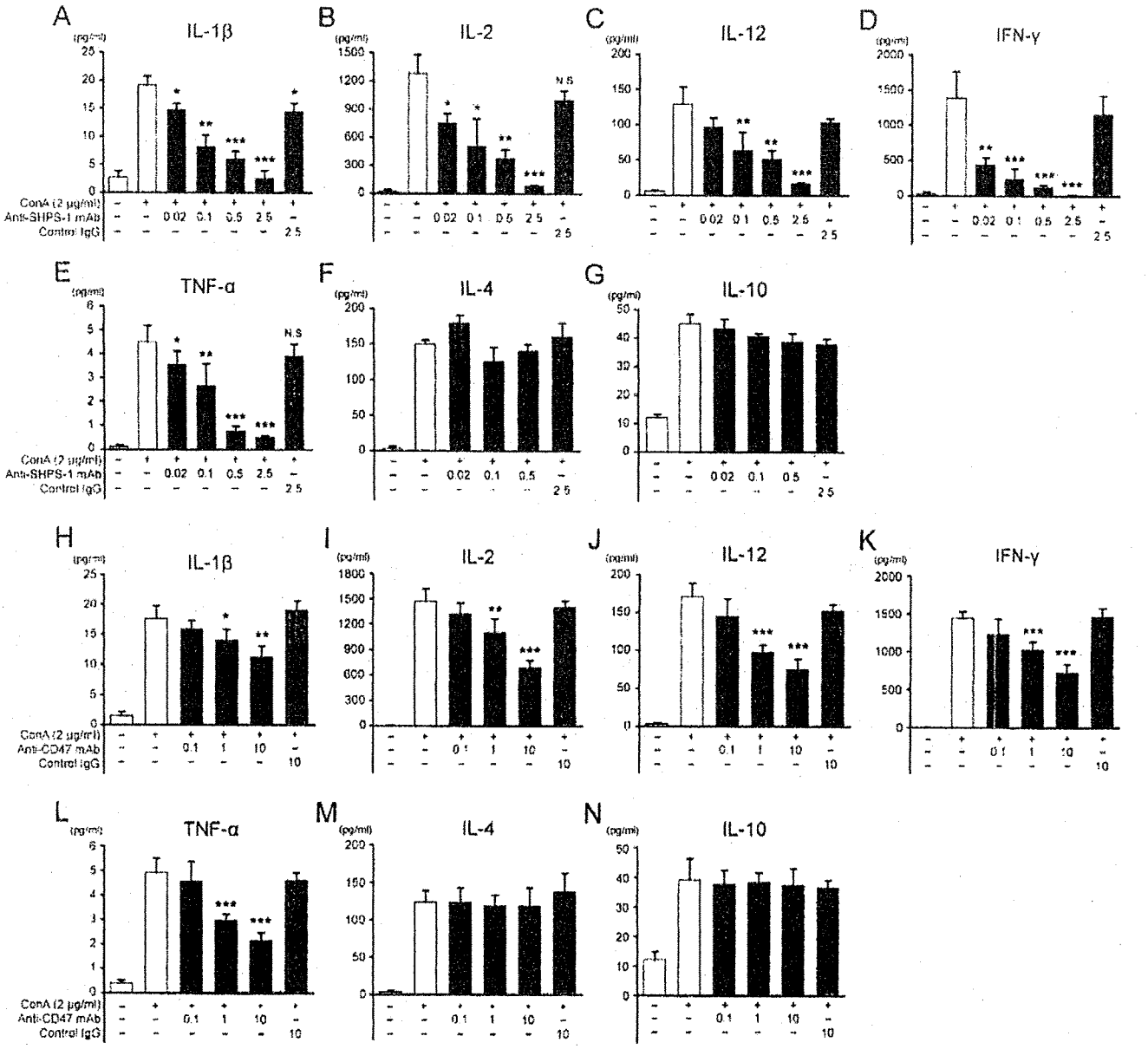

Figure 5. (A-G) Murine spleen cells were stimulated with ConA (2 μg/ml) in media containing graded doses of anti-SHPS-1 mAb or control IgG (2.5 μg/ml). 24 h later, supernatants were collected, and concentrations of IL-1ß (A), IL-2 (B), IL-12 (C), IFN-γ (D), TNF-α (E), IL-4 (F), and IL-10 (G) were determined by ELISA. (H-N) Experiments were repeated with anti-CD47 mAb, and concentrations of IL-1ß (H), IL-2 (I), IL-12 (J), IFN-γ (K), TNF-α (L), IL-4 (M), and IL-10 (N) were determined. Experiments were repeated 3 or 4 times. Data are mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control IgG.

0.079, and 1.18, respectively. Anti-CD47 mAb showed similar effects on cytokine release (Figure 5H-5N). This antibody reduced the concentration of IL-2, IL-12, IFN-γ, and TNF-α in the media in a dose-dependent manner. However, its inhibitory effect was much lower than that of anti-SHPS-1 mAb, and the suppression was no more than 60% even with 10 μg/ml of the antibody.

DISCUSSION

The results of our study demonstrate that the administration of an anti-SHPS-1 mAb successfully reduces the severity of arthritis in CIA mice. CIA is an animal model often used to study the pathology of RA, in which both humoral and cell-mediated immunity is necessary for the development of arthritis[31,32]. The treatment with the anti-SHPS-1 mAb virtually did not suppress the humoral immunity, since it did not alter the concentration of anti-CII antibodies in the sera of mice (Figure 6). Thus, the therapeutic effect of the anti-SHPS-1 mAb could be ascribed entirely to the suppression of the cell-mediated immune response. In *in vitro* experi-

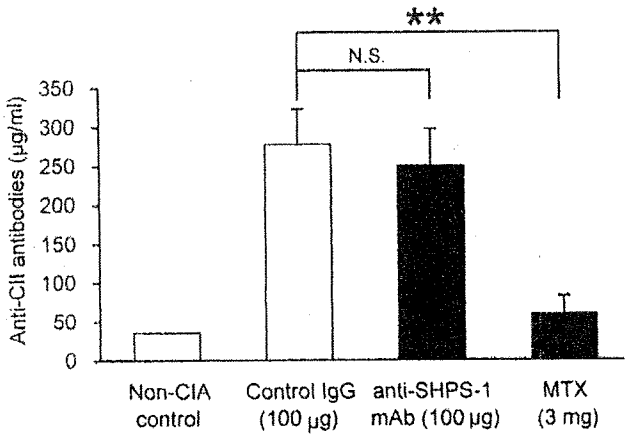

Figure 6. Concentration of anti-CII antibodies in the sera. CIA mice were treated for 11 days with control IgG (100 μg), anti-SHPS-1 mAb (100 μg), or MTX (3 mg), and the sera were obtained. Sera were also obtained from mice in which CIA was not induced, and the concentrations of anti-CII antibodies were determined by ELISA. Data are mean ± SD of 5–8 mice. **$p < 0.01$ compared with control IgG. NS: nonsignificant.

ments using murine spleen cells, addition of the anti-SHPS-1 mAb inhibited the release of IL-1ß, IL-2, IL-12, IFN-γ, and TNF-α into the media upon stimulation by ConA, while the release of IL-4 or IL-10 was almost unaffected. The finding that the anti-SHPS-1 mAb suppressed the release of cytokines primarily from Th1 cells but not those from Th2 cells further supports the idea that the anti-SHPS-1 mAb affects cellular immunity rather than humoral immunity. In human RA, TNF-α is profoundly involved in the progression of the disease as shown by the efficacy of anti-TNF-α therapy[33-35]. Also, IL-2 and IFN-γ are known to be involved in the catabolism in affected joints[36]. Since the pathology of arthritis in the CIA mouse closely resembles that of human RA[37], the reduction in the release of those cytokines could reasonably explain the therapeutic effects of the anti-SHPS-1 mAb observed in this work.

For such change in cytokine release, ligation of SHPS-1 by anti-SHPS-1 mAb may play a significant role, in addition to the inhibitory role of the antibody upon SHPS-1/CD47 interaction. We previously showed that SHPS-1 ligation by the antibody inhibits the migration and maturation of epidermal Langerhans cells, which suggests that DC function could be regulated by SHPS-1 engagement[23,24]. Ligation of SHPS-1 has been shown to inhibit TNF-α production by lipopolysaccharide-stimulated monocytes[38]. Thus, the observed reduction in TNF-α release by anti-SHPS-1 mAb could be ascribed, at least in part, to the suppression of TNF-α production by macrophages or DC by SHPS-1 ligation. Again, since the antibody inhibits IL-12 production by DC[16], the observed suppression of IL-12 release could be partly caused by SHPS-1 ligation. Because IL-12 is an essential cytokine for Th1 development, reduced IL-12 production favors the development of Th2 cells rather than Th1 cells. This is compatible with the finding that the production of all Th1 cytokines, but not those of Th2, was suppressed by anti-SHPS-1 mAb. The difference between anti-SHPS-1 and anti-CD47 mAb in the effects on cytokine release may be reasonable if these direct actions are assumed with the former antibody.

On the other hand, the supposed suppression of cellular immunity by anti-SHPS-1 mAb may be caused primarily by the inhibition of interaction between SHPS-1 and CD47. T cells express CD47 at a high density[39]. Since SHPS-1/CD47 interaction positively regulates T cell responses[21], it is possible that the anti-SHPS-1 mAb suppressed T cell activation by blocking that interaction. Anti-SHPS-1 mAb may inhibit proliferation of T cells via the suppression of TNF-α production by antigen-presenting cells[22]. Other studies have shown that SHPS-1/CD47 interaction may downregulate DC-T cell interaction, by reducing IL-12 production by DC and IL-12 receptor expression on T lymphocytes[16,17,39]. Reduced T cell activation by these mechanisms could be involved in the amelioration of arthritis by anti-SHPS-1 mAb.

Meanwhile, a mechanism for the reduction of bone erosion by the antibody was suggested by an *in vitro* experiment. Our current investigation and that of others consistently indicate that anti-SHPS-1 mAb and anti-CD47 mAb both inhibited induction of osteoclasts from macrophages[40]. Macrophages express SHPS-1 and CD47 abundantly, and utilize them for cell fusion, which is an essential step for osteoclast formation[7,11,20]. Therefore, it is likely that the antibodies for these molecules reduced the formation of osteoclasts through the inhibition of multinucleation. In addition to this, anti-SHPS-1 mAb might have reduced osteoclast formation through the change in released cytokines discussed above: among the cytokines whose release was suppressed by the antibody, IL-1ß and TNF-α are known to play essential roles in the formation of osteoclasts[3,4]. In our study, suppression of osteoclast formation was more obvious with anti-SHPS-1 mAb than with anti-CD47 mAb (Figure 4). This difference, again, could be ascribed to the lack of SHPS-1 ligation with the latter antibody.

Our results show that the use of anti-SHPS-1 antibody could be a promising strategy to treat patients with RA. Although our current results are based on an animal model of RA, the treatment with the antibody seems attractive because the antibody could regulate T cell immunity and osteoclast formation together, both of which are essential in treating RA[3,4,41]. Further studies are awaited to determine the feasibility of the antibody treatment.

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—Review—
Review Series: Animal Bioresource in Japan

Current Activities of CARD as an International Core Center for Mouse Resources

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Abstract: The Center for Animal Resources and Development (CARD), Institute of Resource Development and Analysis, Kumamoto University was established in 1998 based on recommendations published in the report "Preservation, Supply and Development of Genetically Engineered Animals" by the Ministry of Education, Culture, Sports, Science and Technology. We provide a comprehensive and integrated set of research services designed for the mouse-based biological research community. All services are conducted in accordance with the highest standards of animal health and genetic quality and are delivered to meet researcher's research goals. To promote biological sciences worldwide, we produce genetically engineered mice and exchangeable gene trap ES clones, cryopreserve mouse embryos and sperm, supply these resources, organize training courses to educate people, and form a hub of the domestic and international networks of both mutagenesis and resource centers. Up to now, we have produced more than 600 genetically engineered mouse strains and have more than 1,100 strains and stocks of mice for supply to the scientific community. More than 150 studies using genetically engineered mice produced or supplied by CARD have been published so far. As a founding member of the Federation of International Mouse Resources, the Asian Mouse Mutagenesis and Resource Association, and the International Gene Trap Consortium, we are contributing to the promotion of biological sciences in the world.

Key words: cryopreservation, knockout, mouse, resource, transgenic

Introduction

The Center for Animal Resources and Development (CARD), Kumamoto University was established in 1998 based on recommendations published in the report "Preservation, Supply and Development of Genetically Engineered Mice" by the Subdivision of Resource Study, Council for Science and Technology, Ministry of Education, Culture and Science on July 10, 1997. That report recommended the establishment of at least two centers which could preserve, supply, and develop genetically engineered mice in Japan. At the Institute of Molecular Embryology and Genetics, Kumamoto University, we established the Laboratory of Transgenic Technology in 1992 and started to produce transgenic mice in response to requests from scientists. This was the first trial for

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such a service in Japan. The first transgenic mice were produced by Gordon *et al.* in 1980 [4]. Since then, transgenic mice have been used in many biological studies and have been shown to be a rich resource for such studies. In 1989, the first knockout mice were produced using a homologous recombination technique in embryonic stem (ES) cells [8]. With this procedure we aimed to promote scientific activities through the production of genetically engineered mice. In the mid 1990s, we realized that one transgenic mouse or knockout mouse strain can be used in many different areas of biological science, and that there was a strong demand for the preservation and supply of genetically engineered mouse strains. At the same time, the technology for the cryopreservation of mouse embryos had developed enough to be able to respond to such demand. In 2000, a new facility for embryo cryopreservation was completed and we started to cryopreserve, supply, and develop genetically engineered mice.

Goals of CARD

Our goals are as follows: 1) the establishment of mouse resources unique in Asia by integration of advanced technologies in production, cryopreservation, and the supply of genetically engineered mice; 2) the development of technologies relevant to these areas to keep our techniques advanced; 3) the establishment of a supply system between our center and other institutions for cryopreserved embryos or gametes to protect against infection which may arise through the transfer of live mice; 4) the training and education of workers and researchers in this field, especially those involved in reproductive engineering; and 5) the formation of domestic and international networks to promote biological sciences globally. To accomplish these goals, we became a founding member of the Federation of International Mouse Resources (FIMRe) [3]. FIMRe is a collaborating group of Mouse Repository and Resource Centers worldwide whose collective goal is to archive and provide strains of mice as cryopreserved embryos and gametes, ES cell lines, and live breeding stock to the research community. Goals of the FIMRe are (1) Coordinate repositories and resource centers to archive valuable genetically defined mice and ES cell lines being created worldwide and meet research demand for these genetically defined mice and ES cell lines, (2) Establish consistent animal health standards of the highest quality in all resource centers, (3) Provide genetic verification and quality control for genetic background and mutations, (4) Provide resource training to enhance user ability in utilizing cryopreserved resources.

In addition, we organized the Asian Mouse Mutagenesis and Resource Association (AMMRA) in 2006. The AMMRA is a collaborative group of Mouse Mutagenesis and Resource Centers in Asia. Its mission is "To promote mouse mutagenesis projects and to facilitate access to mouse resources in Asia". Its goals are "The use of mouse models for understanding genome function and the improvement of human health". The founding members of AMMRA are (1) Biological Resource Center, Singapore, (2) National Laboratory Animal Center, Taipei, (3) National Resource Center for Mutant Mice, Nanjing University, Nanjing, (4) Shanghai Institute of Biological Sciences, Shanghai, (5) Nanfang Center for Model Organisms, Shanghai, (6) Peking University-BLARC, Beijing, (7) Beijing Institute of Laboratory Animal Science, CAMS, PUC, Beijing, (8) Bio-Evaluation Center, KRIBB, Daejeon, (9) Riken BioResource Center, Tsukuba, (10) Center for Animal Resources and Development, Kumamoto.

Characteristics of the Mouse

Development of the laboratory mouse as a research model really began with genetic experiments in the early 1900s. Today, a large number of inbred strains of mice or various strains of mice with spontaneous or induced mutations (i.e., transgenics, targeted mutations, chemically induced mutations) have been produced in a variety of laboratories worldwide. They are prized for many qualities, including their small size, short generation time, and ease of breeding within the laboratory. The fact that they are genetically the best characterized of all mammals increases their value for all fields of study.

Technical Services Available at CARD

According to our aims, we provide a comprehensive,

Table 1. Production, cryopreservation, supply, and cleaning of genetically engineered mice at CARD since 1998

	Tg	Chimera	Total	No. of strains cryopreserved	No. of strains supplied	No. of strains cleaned
1998	67	6	73			3
1999	22	1	23			53
2000	66	11	77	144	4	62
2001	60	15	75	97	10	73
2002	59	21	80	67	34	47
2003	61	23	84	89	33	20
2004	44	14	58	116	58	65
2005	43	18	61	111	39	83
2006	23	13	36	153	42	83
2007	25	7	32	207	35	48
2008	37	6	43	142	53	106
Total	507	135	642	1,126	308	643

integrated, and highly customizable set of research services designed for the mouse-based biomedical research community. All services are conducted in accordance with the highest standards of animal health and genetic quality and are delivered to meet user's schedule and research goals. The intellectual property right of each resource belongs to the depositor, not to our center. Users should consider this issue before submitting a request for resources, and undertake signed agreements to any conditions attached to a resource.

(1) Production of genetically engineered mice

Upon requests by researchers, we produce either transgenic mice or chimeric mice. So far, we have produced 507 transgenic mouse lines and 135 chimeric mouse lines (Table 1). Researchers should prepare and send DNA constructs for microinjection into fertilized eggs. We usually use fertilized eggs obtained from C57BL/6 mice. We microinject DNA into fertilized mouse eggs and transfer them into oviducts of foster mothers. We rear new born mice up to 4 weeks of age and then send these mice to requesters. So, researchers need to carry out screening for transgenic mice at their facilities. For chimeric mouse production, researchers should prepare knockout ES clones at their facilities. We make chimeric mice by injecting targeted ES cells into blastocysts, which are then transferred to the uteri of foster mothers. We send all chimeric mice to requesters at 4 weeks of age. It should be noted that researchers should have permission for recombinant DNA experiments at their institutions.

(2) Cryopreservation of embryos and sperm

For deposit, researchers should send their mice to CARD. The numbers of mice that should be shipped to CARD for embryo freezing is as follows. For genetically engineered mouse strains, researchers should send at least 3 homozygous or 3 heterozygous male mice. Alternatively, researchers can send at least 5 homozygous males and 20 homozygous females if they desire cryopreservation of homozygous embryos. For other mouse strains, we need at least 5 males and 20 females for cryopreservation.

We generally produce embryos from these mouse strains using *in vitro* fertilization. Ten straws containing spermatozoa and 8 tubes containing 40 two-cell embryos are cryopreserved for each strain. We use the simple vitrification method for embryo freezing [6] and the Nakagata method for sperm freezing [5]. For quality control, some of the frozen embryos are thawed to check if the frozen embryos develop into viable young and whether or not the developed mice are microbiologically clean. For genetically engineered mouse strains, transgene transmission is confirmed by PCR analysis of genomic DNA from tail tissue of the produced mice.

Currently our center has 1,126 strains and stocks of mice. These include inbred mouse strains, spontaneous

mutants, transgenic mice, and knockout mice. CARD R-BASE (Resource Database) provides resource-related strain, gene and reference information (http://cardb.cc.kumamoto-u.ac.jp/transgenic/index.jsp).

(3) Supply of mouse strains and frozen embryos/sperm

Cryopreserved embryos or recovered populations from cryopreserved embryos are supplied to the scientific community. The cost is141,750 JY or 109,200 JY for supply of recovered mice or frozen embryos, respectively. The application procedure for the supply of laboratory mice is shown on our web page (http://card.medic.kumamoto-u.ac.jp/card/english/sigen/gyoumu/ebank/index.htm). We have already supplied 90 strains of cryopreserved embryos and 218 strains of mice worldwide.

(4) Exchangeable gene trap clones (EGTC)

Our center has more than 600 exchangeable gene trap ES clones. In general, gene trapping is a high-throughput approach that is used to introduce insertional mutations across the genome in mouse embryonic stem (ES) cells. In addition to generating standard loss-of-function alleles, newer gene trap vectors offer a variety of post-insertional modification strategies for the generation of other experimental alleles. We are using the exchangeable gene trap method for isolation of gene trap clones [1, 2, 9]. This method has a great advantage. The exchangeable gene trap vector is usually inserted around the exon containing the ATG codon in a mouse endogenous gene, resulting in the null mutation. As the β-geo gene is flanked by lox71 and loxP, the β-geo gene can be replaced with the gene of interest by electroporating a new vector containing the gene of interest flanked by lox66 and loxP together with a Cre expression vector. The newly introduced gene of interest can be expected to be expressed under the control of the trapped gene. Using this system, a mouse gene can be replaced with a homologous human gene, leading to the production of a humanized mouse at the gene level. These clones can be accessed through the EGTC database (http://egtc.jp/view/index). As we are also the founding member of the international gene trap consortium (IGTC) [7], the EGTC database is connected to the integrated database of the IGTC. The IGTC database contains all publicly available gene trap cell lines, which are available on a non-collaborative basis for nominal handling fees. Researchers can search and browse the IGTC database for cell lines of interest using accession numbers or IDs, keywords, sequence data, tissue expression profiles, and biological pathways. At the moment, the IGTC database has 380,863 cell lines. IGTC members are BayGenomics (USA), Centre for Modelling Human Disease (Toronto, Canada), Embryonic Stem Cell Database (University of Manitoba, Canada), German Gene Trap Consortium (Germany), Sanger Institute Gene Trap Resource (Cambridge, UK), Soriano Lab Gene Trap Database (Mount Sinai School of Medicine, New York, USA), Texas Institute for Genomic Medicine—TIGM (USA), TIGEM-IRBM Gene Trap (Naples, Italy), and our institution, Exchangeable Gene Trap Clones (Kumamoto University, Japan).

(5) Other resources

In the Gene Technology Center, which also belongs to the Institute of Resource Development and Analysis, we started the GTC P-stock service in April 2004. This is not a plasmid bank; however, we do store valuable transgenes or targeting constructs for homologous recombination and supply them upon request (http://gtc.egtc.jp/view/pstock/index).

(6) Courses and education

CARD sponsors stimulating courses and conferences on our campus at Honjo in Kumamoto and at other locations inside and outside Japan. CARD training courses for reproductive engineering techniques were introduced in November 2000. Spring and autumn courses are staged each year at CARD. The courses teach the following techniques:1) Preparing and Assembling Pipettes for Embryo Handling, 2) *In Vitro* Fertilization, 3) Simple Vitrification of Mouse Embryos, 4) Vitrification and Transplantation of Mouse Ovaries, 5) Cryopreservation of Mouse Spermatozoa, 6) *In Vitro* Fertilization using Cryopreserved Spermatozoa, 7) Collecting Two-Cell-Stage Embryos, 8) Vasectomy for the Creation of Sterile Males, 9) Embryo Transfer into the Oviduct, 10) Production of Chimeric Mice by 8-Cell Aggregation, 11) Embryo Transfer into the Uterus, and 12) Caesarean Section and Fostering. We sometimes hold the training course at other venues inside and outside Japan.

Popular Strains: Characteristic Features and Their Use for Research

Table 2 shows the mouse strains which are requested by many researchers. A recent trend is that requests for Cre mice are increasing substantially due to the performance of its conditional knockout.

Among Cre mice, P0-Cre is one of the most frequently requested strains. Although this strain of mouse has been described in a published manuscript [10], we would like to briefly introduce the characteristics of this mouse. Neural crest cells are embryonic, multipotent stem cells that give rise to various cell/tissue types and thus serve as a good model system for the study of cell specification and mechanisms of cell differentiation. For analysis of neural crest cell lineage, transgenic mice harboring a Cre gene driven by a promoter of protein 0 (P0) were generated. To detect and visualize the Cre-mediated DNA recombination in neural crest cells and derivatives, we utilized another transgenic (Tg) line with the CAG-CAT-Z indicator construct [10]. This Tg line carries a *lacZ* reporter gene downstream of a chicken β-actin promoter and a "stuffer" fragment flanked by two *loxP* sequences. The *lacZ* is expressed only when the stuffer is removed by the action of Cre recombinase. Using these transgenic mice, we demonstrated that a subset of migrating neural crest cells and a wide variety of cells in the neural crest cell lineage could be marked by *lacZ* expression. Thus, the use of this system may facilitate many interesting experiments, including lineage analysis, purification, and manipulation of the mammalian neural crest cells. Also, this cell-type-specific transgenesis system should facilitate functional analysis of genes of interest in the neural crest cell lineage. Up to now, this strain of mouse has been used in many studies and is now one of the most useful Cre-driver mice.

Deposition and Request for Mouse Strains and EGTC

An outline of the deposition and request procedures for mouse strains is illustrated in Fig. 1. Information on the application procedure is available on the web page (Fig. 2) (http://card.medic.kumamoto-u.ac.jp/card/english/index.html). As we are one of the founding members of FIMRe (http://www.fimre.org/), we also offer our mouse data to the integrated database IMSR (International Mouse Strain Resources) (http://www.informatics.jax.org/imsr/index.jsp). Thus, researchers also can find mouse strains using these databases.

Table 2. Frequently requested mouse strains

ID: 148	C57BL/6J-Tg(PO-Cre)94Imeg
ID: 250	Tg(K5-Cre)
ID: 709	B6;C3H-Tg(K19-Wnt1/K19-Ptgs2/K19-Ptges)
ID: 428	C57BL/6N-Tg(CAG-AURKA(WT)Card
ID: 196	B6;129-Synd4^{tm1}
ID: 189	Tg(E/nestin:EGFP-50)
ID: 175	Tg(E/nestin:EGFP-25)
ID: 91	Tg(CAG-Cre)
ID: 89	Tg(lck-Cre)
ID: 509	B6;D2-Tg(CAG-CAT-EGFP)39Miya
ID: 355	C57BL/6-CD9^{tm1};Tg(ZP3-EGFPCD9)
ID: 312	B6;CB-Dtr$^{tm2(lox)}$
ID: 290	C57BL/6 -Tg(Act-EGFP)C14-Y01-FM131Osb
ID: 88	C57BL/6-Rag1$^{tm1(GFP)Imku}$

Major Achievement: Publication

More than 150 studies using genetically engineered mice produced by CARD, have been published so far. Among these some representative manuscripts are listed in Table 3. This list clearly suggests that technical services in mouse embryo manipulation and reproductive engineering contribute greatly to the promotion of science.

Related Information

JMSR (http://www.shigen.nig.ac.jp/mouse/jmsr/top.jsp): The Japan Mouse/Rat Strain Resources Database (JMSR) is a searchable online database of mouse/rat strains and stocks available in Japan. The JMSR was developed in 2001 through discussions with the Mouse Genetic Resources Subcommittee (http://www.shigen.nig.ac.jp/shigen/grc/grc_mouse.jsp). The objective of this database is to provide a portal site which will help users to locate and obtain mouse/rat resources. This site is maintained by the Genetic Informatics Laboratory, National Institute of Genetics in close cooperation with data providers.

IGTC (http://www.genetrap.org/): The International

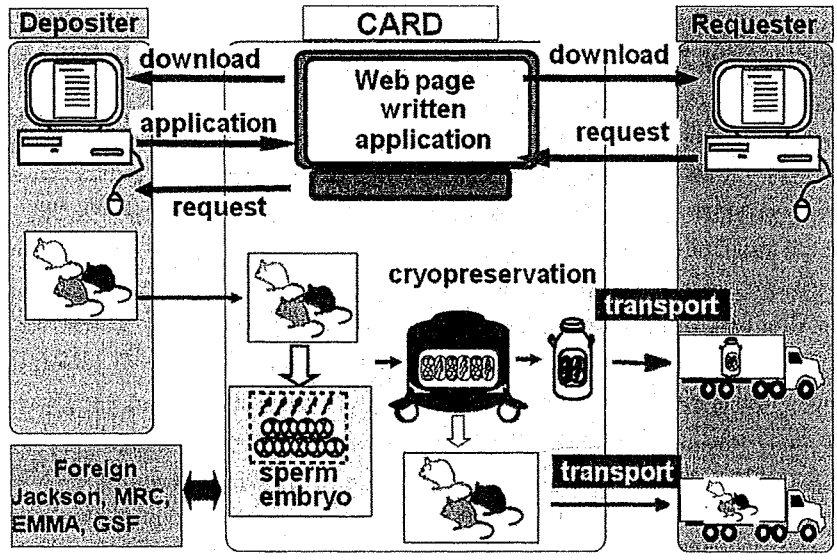

Fig. 1. Outline of deposition and request procedures for mouse strains. Information on the application procedure and necessary documents are available on the web page. Mouse embryos or sperm obtained from mice sent from depositors are cryopreserved and stored in liquid nitrogen tanks. Frozen embryos or live mice obtained by transfer of thawed embryos into foster mothers can be sent to requesters.

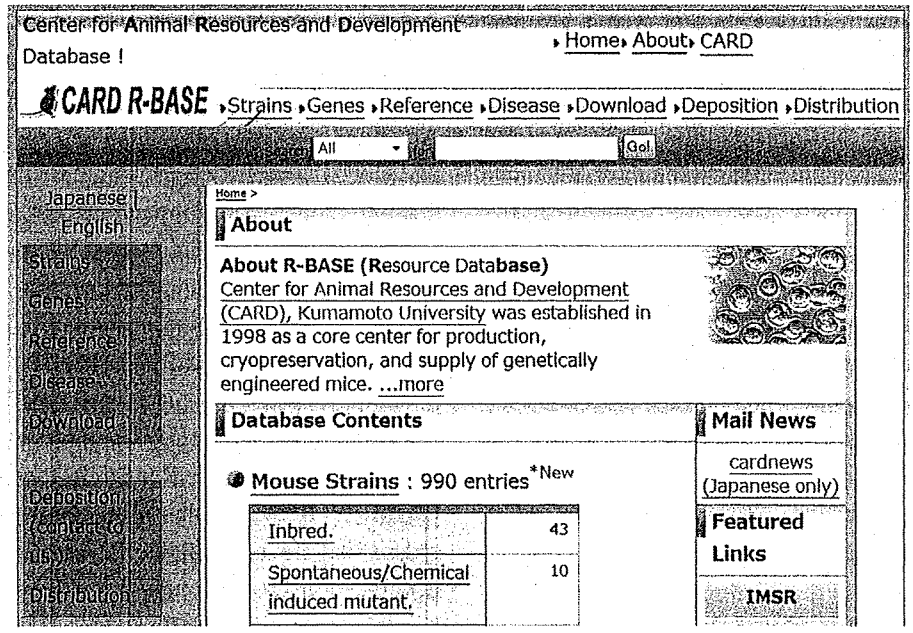

Fig. 2. Web page of CARD R-BASE. Only part of the web page is shown in this figure. Both Japanese and English versions are available.

Gene Trap Consortium (IGTC) consists of laboratories around the world working together to generate a public library of mutated murine ES cell lines. Such cell lines can be obtained on a non-collaborative basis by scientists interested in generating reporter-tagged, loss-of-function mutations in mice. In addition to loss of function, new-

Table 3. Selected publications

Ohnishi, N. *et al. Proc. Natl. Acad. Sci. U.S.A.* 105: 1003–1008, 2008.
Sato, T. *et al. Nature* 448: 366–369, 2007.
Imai, T. *et al. Science* 314: 657–661, 2006.
Serizawa, S. *et al. Cell* 127: 1057–1069, 2006.
Shim, J.H. *et al. Genes Dev.* 19: 2668–2681, 2005.
Terada, K. *et al. EMBO J.* 24: 611–622, 2005.
Tachibana, M. *et al. Genes Dev.* 19: 815–826, 2005.
Ohmuraya, M. *et al. Gastroenterology* 129: 696–705, 2005.
Itoh, H. *et al. Gastroenterology* 127: 1423–1435, 2004.
Yamazaki, S. *et al. J. Cell Biol.* 163: 469–475, 2003.
Ishida, D. *et al. Cancer Cell* 4: 55–65, 2003.
Serizawa, S. *et al. Science* 302: 2088–2094, 2003.
Wakabayashi, Y. *et al. Nat. Immunol.* 4: 533–539, 2003.
Yokosuka, T. *et al. J. Exp. Med.* 195: 991–1001, 2002.
Pepys, M.B. *et al. Nature* 417: 254–259, 2002.
Tachibana, M. *et al. Genes Dev.* 16: 1779–1791, 2002.
Yamauchi, T. *et al. Nat. Genet.* 30: 221–226, 2002.
Hasegawa, S. *et al. Proc. Natl. Acad. Sci. U.S.A.* 99: 297–302, 2002.
Yamaguchi, S. *et al. Nature* 409: 684, 2001.
Nishimura, H. *et al. J. Exp. Med.* 191: 157–169, 2000.
Hisahara, S. *et al. EMBO J.* 19: 341–348, 2000.
Nakai, A. *et al. EMBO J.* 19: 1545–1554, 2000.
McIlroy, D. *et al. Gene. Dev.* 14: 549–558, 2000.
Serizawa, S. *et al. Nat. Neurosci.* 3: 687–692, 2000.
Ishiguro, K. *et al. J. Clin. Invest.* 106: 873–878. 2000.
Shinkura, R. *et al. Nat. Genet.* 22: 74–77, 1999.
Terauchi, Y. *et al. Nat. Genet.* 21: 230–235, 1999.
Watanabe, N. *et al. J. Exp. Med.* 190: 461–469, 1999.
Ihara, Y. *et al. Proc. Natl. Acad. Sci. U.S.A.* 95: 2526–2530, 1998.
Fujii, H. *et al. EMBO J.* 17: 6551–6557, 1998
Watanabe, D. *et al. Cell* 95: 17–27, 1998.
Sawada, S. *et al. J. Exp. Med.* 187: 1439–1449, 1998.

er gene trap vectors offer a variety of post-insertional modification strategies to allow for the generation of other experimental alleles. The cooperative goal of the IGTC is to generate an international resource representing all or most genes in the mouse genome, and to provide the bioinformatics and logistical support to make the resource valuable and available to scientists.

IMSR (http://www.informatics.jax.org/imsr/index.jsp): The International Mouse Strain Resources (IMSR) is a searchable online database of mouse strains and stocks available worldwide, including inbred, mutant, and genetically engineered mice. The goal of the IMSR is to assist the international scientific community in locating and obtaining mouse resources for research. The data content found in the IMSR is as it was supplied by data provider sites.

EUCOMM (http://www.eucomm.org/): The European Conditional Mouse Mutagenesis Integrated Project (EUCOMM) is funded by the European Union Framework 6 programme. The goal of EUCOMM is to generate a collection of up to 13,000 mutated genes in mouse C57BL/6N embryonic stem (ES) cells using conditional gene trapping and gene targeting approaches. This library will enable mouse mutants to be established worldwide in a standardized and cost-effective manner, making mouse mutants available to a much wider biomedical research community than has been possible previously.

NorCOMM (http://norcomm.phenogenomics.ca/): NorCOMM (North American Conditional Mouse Mutagenesis project) is a large-scale research initiative focused on developing and distributing a library of mouse embryonic stem (ES) cell lines carrying single conditional knockout mutations across the mouse genome.

KOMP (http://www.nih.gov/science/models/mouse/knockout/): The Knockout Mouse Project is a trans-NIH initiative that aims to generate a comprehensive and public resource comprised of mouse embryonic stem (ES) cells containing a null mutation in every gene in the mouse genome.

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Establishment of germline-competent embryonic stem cell lines from the MSM/Ms strain

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Abstract MSM/Ms is an inbred mouse strain established from the Japanese wild mouse, *Mus musculus molossinus*, which has been phylogenetically distinct from common laboratory mouse strains for about 1 million years. The nucleotide substitution rate between MSM/Ms and C57BL/6 is estimated to be 0.96%. MSM/Ms mice display unique characteristics not observed in the commonly used laboratory strains, including an extremely low incidence of tumor development, high locomotor activity, and resistance to high-fat-diet-induced diabetes. Thus, functional genomic analyses using MSM/Ms should provide a powerful tool for the identification of novel phenotypes and gene functions. We report here the derivation of germline-competent embryonic stem (ES) cell lines from MSM/Ms blastocysts, allowing genetic manipulation of the *M. m. molossinus* genome. Fifteen blastocysts were cultured in ES cell medium and three ES lines, Mol/MSM-1, -2, and -3, were established. They were tested for germline competency by aggregation with ICR morulae and germline chimeras were obtained from all three lines. We also injected Mol/MSM-1 ES cells into blastocysts of ICR or C57BL/6 × BDF1 mice and found that blastocyst injection resulted in a higher production rate of chimeric mice than did aggregation. Furthermore, Mol/MSM-1 subclones electroporated with a gene trap vector were also highly efficient at producing germline chimeras using C57BL/6 × BDF1 blastocyst injection. This Mol/MSM-1 ES line should provide an excellent new tool allowing the genetic manipulation of the MSM/Ms genome.

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Introduction

Mus musculus is divided into at least four major subspecies: *M. m. domesticus*, *M. m. bactrianus*, *M. m. musculus*, and *M. m. castaneus* (Bonhomme and Guénet 1996; Moriwaki et al. 1994). An additional subspecies, known as *M. m. molossinus*, is found in Japan. This is not an independent subspecies but arose through hybridization between *M. m. musculus* and *M. m. castaneus* (Yonekawa et al. 1988). MSM/Ms is a mouse strain derived from Japanese *M. m. molossinus* wild mice collected in 1978 in Mishima, Japan (Moriwaki et al. 1994). This strain has been inbred for 100 generations and can therefore be regarded as an inbred strain of *M. m. molossinus*.

The MSM/Ms strain is widely used for linkage analysis and positional cloning because of its genetic divergence from common laboratory mouse strains that are derived predominantly from *M. m. domesticus*. Recently, an arrayed bacterial artificial chromosome (BAC) library of the MSM/Ms genome has been constructed, and BAC clone-end sequence analysis revealed that 0.96% of the nucleotides in the MSM/Ms genome differed from those in the common laboratory mouse

strain, C57BL/6 (Abe et al. 2004). Considering that the genomic difference between humans and chimpanzees is 1.23% (Fujiyama et al. 2002), this nucleotide difference makes the MSM/Ms strain a useful model for functional genomic studies. Corresponding to these genomic sequence differences, many aspects of the phenotype of MSM/Ms mice are also distinct from those of commonly used laboratory strains; for example, they have smaller body size, resistance to carcinogenesis (Miyashita and Moriwaki 1987; Nakanishi et al. 2007; Okumoto et al. 1995), high locomotive activity (Koide et al. 2000), and resistance to high-fat-diet-induced diabetes (Kobayashi et al. 2004). It is therefore expected that genetic studies using MSM/Ms will be able to provide new clues to gene function that cannot be obtained from studies with common laboratory strains. However, genetic manipulation of the MSM/Ms genome has been difficult due to their poor response to superovulation and the absence of an embryonic stem (ES) cell line for this strain.

In this study, we have established three ES cell lines, Mol/MSM-1, -2, and -3, from MSM/Ms embryos and have devised an efficient way to produce germline chimeras by injecting ES cells into blastocysts obtained from mating C57BL/6J female and BDF1 male mice (B6 × BDF1).

Materials and methods

Culture medium

Mouse embryos were cultured using KSOM medium (Lawitts and Biggers 1993) (ARK-Recource Co. Ltd., Kumamoto, Japan). ES cells were cultured in KSR-GMEM medium consisting of Glasgow Minimum Essential Medium (GMEM) (Sigma, St Louis, MO) with 1 × MEM nonessential amino acids (Gibco Invitrogen, Grand Island, NY), 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1% fetal bovine serum (FBS) (HyClone, Thermo Fisher Scientific Inc., Waltham, MA), 14% Knockout™ Serum Replacement (KSR) (Gibco Invitrogen), and 1100 U/ml leukemia inhibitory factor (LIF) (ESGRO, Chemicon, Temecula, CA). For neutralization of trypsin, FBS-GMEM was used in which the KSR in KSR-GMEM was replaced with FBS (final 15% FBS).

Mice and embryos

MSM/Ms morula-stage embryos were collected from the oviducts of naturally mated MSM/Ms females, cryopreserved using the vitrification method (Nakao et al. 1997) at RIKEN BRC, and sent to Kumamoto University. They were quickly thawed according to the method reported by Nakao et al. (1997) and cultured in KSOM (Lawitts and Biggers 1993) for 1 day until they reached blastocyst stage.

F1 hybrid BDF1 (C57BL/6 × DBA2) male mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). C57BL/6J and CD-1 female mice were purchased from CLEA Japan (Tokyo, Japan) and Charles River Laboratories, Inc. (Wilmington, MA), respectively.

To obtain morulae and blastocysts, C57BL/6J and CD-1 females were induced to superovulate by injection of 5 IU PMSG (pregnant mare's serum gonadotropin) (ASKA Pharmaceutical Co. Ltd., Tokyo, Japan), followed 48 h later by injection of 5 IU of hCG (human chorionic gonadotropin) (ASKA Pharmaceutical Co. Ltd.). They were then mated with BDF1 and CD-1 males, respectively. Two-cell-stage embryos were collected from the oviducts of females 42 h after hCG injection and cultured in KSOM until the morula (for 24 h) or blastocyst stages (for 48 h).

Establishment of ES cell line

ES cells were cultured at 37°C in a humidified atmosphere of 6.5% CO_2 in air. MSM/Ms blastocysts were plated individually on a 48-well plate coated with 0.15% gelatin in KSR-GMEM medium. The blastocysts were allowed to hatch and attach to the dish and were refed every 3 days with KSR-GMEM medium. After 10 days in culture, the inner cell mass (ICM) outgrowth was dissociated in threefold-diluted 0.25% trypsin/1 mM EDTA (Sigma), and then plated onto a 24-well plate with a feeder layer of mitomycin C-treated primary mouse embryo fibroblasts. After this first passage, the ES cells were gradually plated onto larger culture plates with feeder layers. ES cells were routinely passaged and diluted five- to sixfold every 2 days, and the medium was changed on alternate days.

Characterization of ES cell lines

ES cells were stained for alkaline phosphatase activity using an Alkaline Phosphatase Staining kit (Sigma) according to the protocol supplied by the manufacturer. Karyotype analysis was performed as described previously (Robertson 1987).

Two polymorphic markers, *D18Mit145* and *D14Mit196*, which distinguish between C57BL/6 and MSM/Ms [Mouse Microsatellite Data Base of Japan (MMDBJ), http://www.shigen.nig.ac.jp/mouse/mmdbj/top.jsp], were used to examine genomic DNA from the established ES cells, a C57BL/6J mouse, TT2 ES cells, E14Tg2a ES cells, and an MSM/Ms mouse, according to the protocol obtained from MMDBJ. The sequences of the primer pairs and the product sizes in C57BL/6, 129 Sv/J, and MSM/Ms were as follows: *D18Mit145* (primers: TTCAGGTGCACCACCAAGTT, CTCCGTCAAAGAAAATGTTAAATC; product size: C57BL/6, 201 bp; 129 Sv/J, 202 bp, MSM/Ms, 269 bp) and *D14Mit196* (primers: CAGGCACAAACAAGTGCTGT, GTGAGTTCTA

GGACATCCCAGG; product size: C57BL/6, 230 bp; 129SV/J, 232 bp; MSM/Ms, 319 bp).

To determine the sex of established ES cells, PCR detecting the *Sry* gene was performed with the primer pair, Sry-F: TGACTGGGATGCAGTAGTTC and Sry-R: TGTGCTAGAGAGAAACCCTG. The size of PCR product was 0.23 kb.

Electroporation

The details of the trap vector pU-21T will be described elsewhere. The main elements of these vectors are the splice acceptor site of the mouse *En-2* gene, and the *βgeo* gene (Taniwaki et al. 2005). Cells transfected with these vectors become G418-resistant. For electroporation, 20–30 μg of *Spe*I-digested plasmid DNA was used. ES cells ($2–3 \times 10^6$ cells) were suspended in 0.8 ml phosphate-buffered saline, electroporated using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) set at 800 V and 3 μF, and then fed with KSR-GMEM medium supplemented with 200 μg/ml G418 after 48 h. Selection was maintained for 7 days, after which the colonies were counted, picked, and placed in 24-well plates.

Production of chimeric mice and confirmation of germline transmission

Chimeric mice were produced by aggregation of ES cells with 8-cell embryos of ICR or B6 × BDF1 mice, and also by injection of ES cells into blastocysts of ICR or B6 × BDF1 mice, as described elsewhere (Nagy et al. 2003). Germline transmission was confirmed by the coat color of F1 offspring. Male chimeras produced from ICR and B6 × BDF1 embryos were mated with ICR and with C57BL/6J or JF1/Ms females, respectively. If no pregnancy was observed within 2 months of mating, in vitro fertilization was performed. For in vitro fertilization, unfertilized eggs were collected from superovulated ICR or C57BL/6J females, and insemination and transfer of fertilized embryos were performed as described previously (Nagy et al. 2003). Because MSM/Ms mice are wild-type with respect to the agouti and albino loci (Aw/Aw, Tyr+/Tyr+), only the F1 progeny derived from MSM/Ms ES cells should exhibit agouti coat color, both in pairings of ICR chimeras with ICR (Tyrc/Tyrc) mice and B6 × BDF1 chimeras with either C57BL/6J (a/a, Tyr+/Tyr+) or JF1/Ms (a/a, Tyr+/Tyr+) mice.

Results

Derivation of ES cells from MSM/Ms blastocysts

Fifteen blastocysts were cultured in KSR-GMEM medium for 10 days, and three ICM-derived colonies were successfully passaged into 10-cm dishes, stocked, and designated Mol/MSM-1, -2, and -3. These first stocks corresponded to passage number (p) 5. The morphologies of the three established ES lines at p7 are shown in Fig. 1a. The appearances of the ES cells were not uniform; they consisted of a mixture of tight, thick colonies and separate, flat colonies. Both populations stained positively for alkaline phosphatase, indicating that

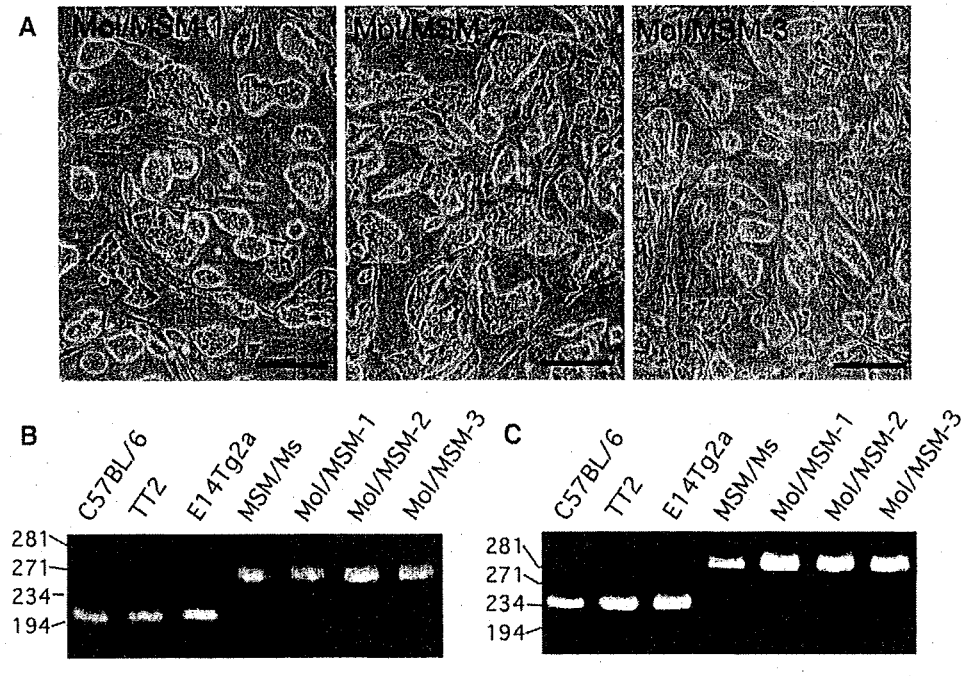

Fig. 1 Generation of Mol/MSM ES lines. **a** Phase contrast photographs of ES lines (p7) stained for alkaline phosphatase activity. Scale bar = 250 μm. **b, c** The genomic DNAs of established ES lines and control genomic DNAs from a C57BL/6J mouse, TT2 ES cells, E14Tg2a ES cells, and an MSM/Ms mouse were examined using two microsatellite markers, *D18Mit145* (**b**) and *D14Mit196* (**c**). Mol/MSM ES cells gave a 269-bp band in (**b**) and a 319-bp band in (**c**) which is characteristic of MSM/Ms. Positions of size marker bands are indicated on the left side

they were undifferentiated. These two types of morphology were still observed after several times of passage and also colony isolation, although the percentage of the two populations varied among subclones. The genomes of the established ES lines were examined using two microsatellite markers, *D18Mit145* and *D14Mit196*, and band sizes characteristic of MSM/Ms were demonstrated, as shown in Fig. 1b, c. The sex of the established ES lines was also examined by genomic PCR detecting the *Sry* gene on the Y chromosome, and all lines were *Sry*-positive, meaning that they were male ES lines (data not shown). For further characterization, metaphase spreads were prepared from the three ES lines and chromosome numbers were counted. As shown in Fig. 2a, over 90% of cells from the Mol/MSM-1 and -3 lines showed normal 40 chromosome numbers, whereas 18% of cells in the Mol/MSM-2 line were tetraploid.

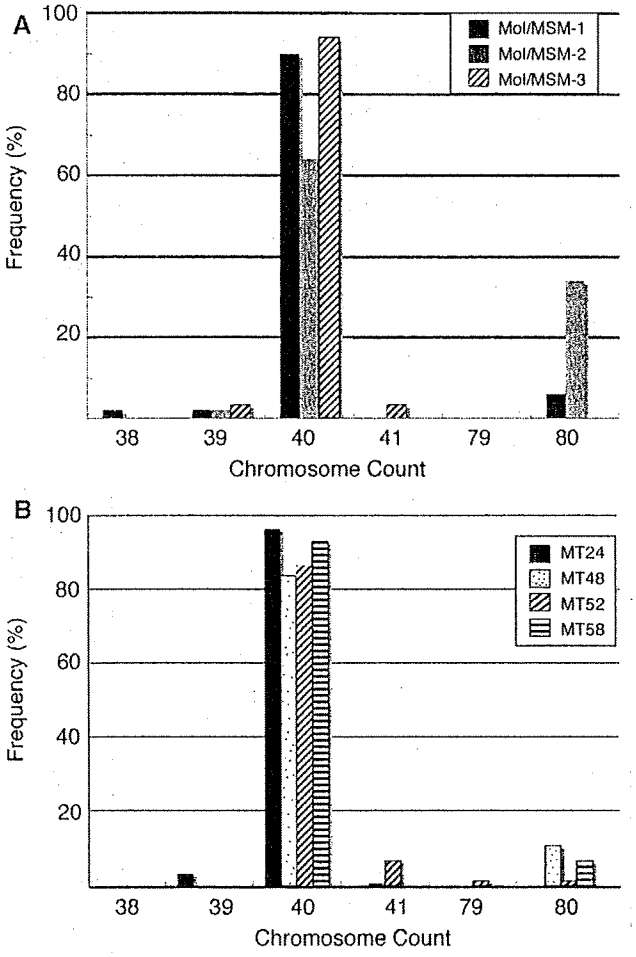

Fig. 2 Chromosome counts of Mol/MSM cell lines and subclones. **a** Chromosome counts of Mol/MSM-1, -2, and -3 lines at p8. **b** Chromosome counts of gene trap clones obtained from Mol/MSM-1. Metaphase spreads of ES cells were prepared, and 50 metaphase spreads were counted for each cell line

Chimeric mouse production and germline transmission

To compare chimera production efficiency, the three ES lines were aggregated with ICR morulae (Table 1). All ES lines resulted in production of male chimeric mice with 100% contribution of ES cells, as shown by coat color. All of the 100% chimeras were able to pass the ES cell genome onto the next generation. The Mol/MSM-1 line showed the highest efficiency of chimera production among the three lines, therefore this line was chosen for subsequent experiments.

To test the stability of the pluripotency, Mol/MSM-1 ES cells were passaged seven times from the first stock without feeder layers (corresponding to p12) and then subjected to aggregation with ICR morulae. As shown in Table 1, the rate of chimera production from these cells was similar to that of earlier passage cells, indicating that Mol/MSM-1 ES cells could be maintained stably.

Injection into blastocysts is a common and orthodox method for the production of chimeras from most ES cell lines. The Mol/MSM-1 line was therefore tested for chimera production by injection into blastocysts derived from ICR or B6 × BDF1 mice. Because mating with BDF1 males could produce higher fertilization rates than mating with C57BL/6 males, and the number of embryos obtained from C57BL/6 females was much larger after mating with BDF1 males than with C57BL/6 males, B6 × BDF1 embryos were used for chimera production. As shown in Table 1, injection into blastocysts increased the efficiency of chimera production (26.7% with ICR and 34.3% with B6 × BDF1) compared with aggregation using ICR morulae (19.2%). However, chimeras obtained by ICR blastocyst injection showed a lower degree of chimerism, determined by coat color (30–70%, data not shown), even in germline chimeras. In contrast, most of the chimeras obtained by B6 × BDF1 blastocyst injection demonstrated 90–100% chimerism. The ratio of germline chimeras to total chimeras produced by B6 × BDF1 blastocyst injection was also higher than that produced by ICR blastocyst injection (Table 1). Thus, B6 × BDF1 blastocyst injection was an efficient method for the production of chimeras using Mol/MSM-1 ES cells.

Germline transmission from subclones after electroporation

To evaluate the ability of the Mol/MSM-1 line to maintain germline competency after genetic manipulation, it was subjected to electroporation and subcloning, and the subclones were then tested for chimera production. A gene trap vector carrying a splice acceptor sequence and the *βgeo* gene was electroporated to Mol/MSM-1 cells. In two experiments, 400 colonies were formed and 142 trap clones

Table 1 Germline transmission of Mol/MSM ES lines

ES cell line (passage no.)	Method of chimeric mouse production	No. of transferred embryos	No. of mice born (% of transferred embryos)	No. of weaned chimeras (% of transferred embryos)	No. of germline chimeras/tested chimeras
Mol/MSM-1 (p6)	Aggregation with ICR morulae	125	43 (34.4%)	24 (19.2%)	9/11
Mol/MSM-2 (p6)	Aggregation with ICR morulae	100	15 (15%)	6 (6%)	1/3
Mol/MSM-3 (p6)	Aggregation with ICR morulae	100	16 (16%)	8 (8%)	2/3
Mol/MSM-1 (p12)	Aggregation with ICR morulae	50	13 (26%)	7 (14%)	2/3
Mol/MSM-1 (p7)	Injection into ICR blastocysts	75	36 (48%)	20 (26.7%)	8/14
Mol/MSM-1 (p7)	Injection into B6 × BDF1 blastocysts	70	28 (40%)	24 (34.3%)	15/18

were stocked. The colony-forming efficiency and growth rate of the G418-resistant subclones after colony isolation were similar to those of the conventional ES cell line, TT2 (Araki et al. 1999). Four trap clones were analyzed for chromosome counts to examine whether euploidy was maintained during subcloning. As shown in Fig.2b, all clones harbored over 80% euploid metaphases, suggesting stability of Mol/MSM-1 line.

For the production of chimeras with trap clones, the aggregation method was initially used. However, as shown in Table 2, only 23% (5 of 22) of trap lines could produce germline chimeras by aggregation with ICR morulae. Although the use of B6 × BDF1 morulae improved the ratio of germline-competent trap lines (31%), this method resulted in a very low production rate of germline chimeras (0.28% with ICR and 0.79% with B6 × BDF1). This meant that 357 ICR morulae or 127 B6 × BDF1 morulae were necessary to obtain one germline chimera.

To further analyze the best combination of methods to produce the most efficient germline transmission, we selected two trap lines, MT6 and MT23, that failed to produce germline chimeras by aggregation with either ICR or B6 × BDF1 morulae (Table 3). Injection into ICR or B6 × BDF1 blastocysts was performed using these two trap lines. As shown in Table 3, ICR blastocyst injection failed to produce any germline chimeras, but 2 of 8 or 11 of 19 chimeras obtained from injection into B6 × BDF1 blastocysts showed germline transmission. These results suggested that B6 × BDF1 blastocyst injection was suitable for chimera production, and we therefore selected a further three trap clones (MT13, MT54, and MT58), which produced a small number of newborn pups but no weaned chimeras after aggregation with B6 × BDF1 morulae (Table 4). MT54 failed to produce any live newborn pups, even using 350 morulae. As shown in Table 4, B6 × BDF1 blastocyst injection using all three clones resulted in efficient germline chimera production, with 7 of 8, 9 of 16, and 10 of 16 germline chimeras being obtained from injection with MT13, MT54, and MT58 trap clones, respectively. Overall, the five trap clones tested for B6 × BDF1 blastocyst injection could produce germline chimeras at an average production rate of 11.2%, meaning that only nine blastocysts were required to obtain one germline chimera. These results clearly suggested that injection of trap clones into blastocysts obtained from B6 × BDF1 mice was the most efficient method of producing germline chimeras.

Discussion

In this study we have demonstrated the establishment of germline-competent ES cells from a wild-derived inbred strain of mice, MSM/Ms. In addition, their subclones, obtained through electroporation and drug selection, could also efficiently produce germline chimeras by injection into B6 × BDF1 blastocysts, suggesting the feasibility of using the Mol/MSM-1 line for genetic manipulation. This ES cell line should provide a powerful tool for the functional analysis of genes in *M. m. molossinus*.

Table 2 Chimera production by aggregation using trap ES lines isolated through electroporation and drug selection

Embryo	No. of trap lines tested	No. of lines producing live pups	No. of lines producing male chimeras	No. of lines producing germline chimeras	Average percentage of germline chimeras to transferred embryos (%)
ICR	22	20 (91%)	18 (82%)	5 (23%)	0.28
B6 × BDF1	39	25 (64%)	13 (33%)	12 (31%)	0.79

Trap clones passaged 4–5 times from G418-resistant colonies were used for chimeric production

Table 3 Chimera production with two trap clones by aggregation or injection method using embryos from ICR or B6 × BDF1 mice

Trap lines	Method for production of chimeras	Recipient embryo	No. of transferred embryos	No. of newborn pups (% of transferred embryos)	No. of weaned male chimeras (% of transferred embryos)	No. of germline chimeras (% of transferred embryos)
MT6	Agg	ICR	150	24 (16)	2 (1.3)	0 (0)
	Inj	ICR	70	19 (27)	4 (5.7)	0 (0)
	Agg	B6 × BDF1	150	6 (4)	1 (0.7)	0(0)
	Inj	B6 × BDF1	70	10 (14)	8 (11)	2 (2.9)
MT23	Agg	ICR	75	7 (9.3)	2 (2.7)	0 (0)
	Inj	ICR	70	52 (74)	19 (27)	0 (0)
	Agg	B6 × BDF1	75	2 (2.7)	0 (0)	0 (0)
	Inj	B6 × BDF1	70	34 (49)	19 (27)	11 (16)

Trap clones passaged 4-5 times from G418-resistant colonies were used for chimeric production. All weaned male chimeras were tested for germline transmission

Agg aggregation with morulae, *Inj* injection into blastocysts

Table 4 Chimera production with three trap clones by aggregation or injection method using embryos from B6 × BDF1 mice

Trap lines	Method for production of chimeras	No. of transferred embryos	No. of new born pups (% of transferred embryos)	No. of weaned male chimeras (% of transferred embryos)	No. of germline chimeras (% of transferred embryos)
MT13	Agg	125	2 (1.6)	0 (0)	0 (0)
	Inj	70	10 (14)	8 (11)	7 (7)
MT54	Agg	350	0 (0)	0 (0)	0 (0)
	Inj	70	20 (29)	16 (23)	9 (13)
MT58	Agg	225	1 (0.4)	0 (0)	0 (0)
	Inj	70	21 (30)	16 (23)	10 (14)

Trap clones passaged 4–5 times from G418-resistant colonies were used for chimeric production. All weaned male chimeras were tested for germline transmission

Agg aggregation with morulae, *Inj* injection into blastocysts

We used KSR to establish and maintain Mol/MSM ES cells. KSR has previously been used for the culture of ES cells from C57BL/6 mice by two other groups, who reported an efficiency of about 20% (Cheng et al. 2004; Shimizukawa et al. 2005). The efficiency of ES cell establishment with MSM/Ms blastocysts in our study was also 20% (three lines from 15 blastocysts), indicating that the MSM/Ms strain is permissive for ES cell derivation. However, this value was almost half that of the ES establishment efficiency (45%) found with F1 blastocysts obtained by injecting MSM/Ms spermatozoa into C57BL/6 oocytes (Shinmen et al. 2007). The higher efficiency in this previous study might have been due to hybrid vigor.

We could obtain germline chimeras from the untreated Mol/MSM-1 line using both the aggregation and the injection methods. However, after electroporation and subcloning, only injection into B6 × BDF1 blastocysts was efficient and was therefore considered to be the more promising method for producing germline chimeras. The decreased efficiency of chimera production using the aggregation method could be due to a reduction in the developmental potential of Mol/MSM-1 ES cells after subcloning. It is known that chimeric mice produced using 8-cell-stage embryos showed higher ES cell contributions than chimeras produced by blastocyst injection (Peli et al. 1996; Tokunaga and Tsunoda 1992). The Mol/MSM-1 ES cell line also showed a similar tendency in that most aggregation chimeras demonstrated 100% chimerism in coat color, while in injection chimeras using B6 × BDF1 blastocysts, 100% chimeras were scarce, and most chimeras showed only 60–90% chimerism. If a subclone with reduced developmental potential was used for aggregation and 100% chimeras were generated with such cells, the chimeras could be lost during prenatal or perinatal development, similar to the instances of perinatal death observed in tetraploid aggregation chimeras (Nagy et al. 1990). The effect of multiple electroporations on germline transmission is currently being investigated. Another reason for the low frequency of germline chimeras produced by the aggregation method is that B6 × BDF1 morulae seem to be more sensitive to treatment with acidic Tyrode's solution, which was used to remove the zona pellucida.