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, CT CCGTCAAAGAAAATGTTAAATC; 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 × 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

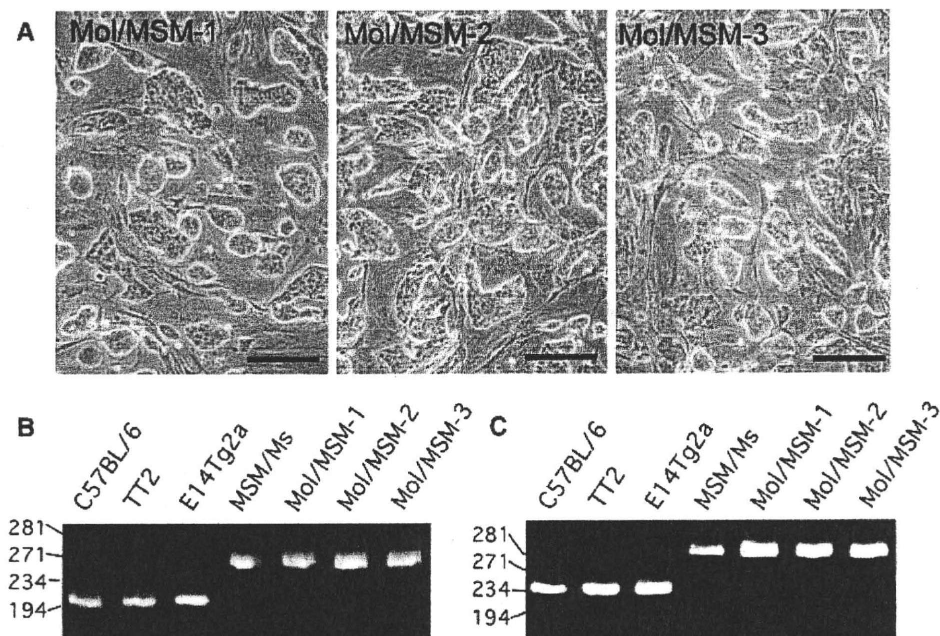

Fig. 1 Generation of Mol/MSM ES lines. **a** Phase contrast photographs of ES lines (p7) stained for alkaline phosphatase activity. Scale bar = 250 μm. **b, c** The genomic DNAs of established ES lines and control genomic DNAs from a C57BL/6J mouse, TT2 ES cells, E14Tg2a ES cells, and an MSM/Ms mouse 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.

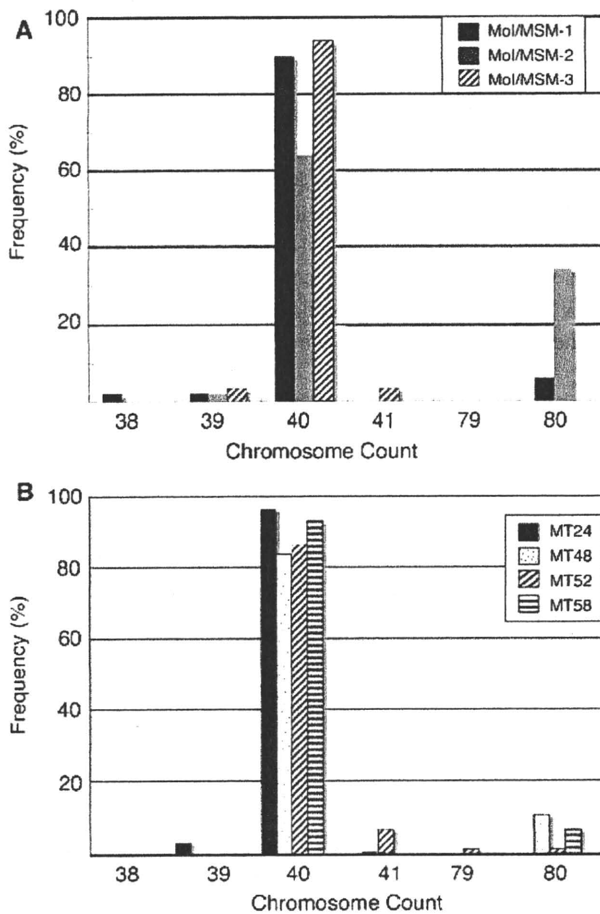

Fig. 2 Chromosome counts of Mol/MSM cell lines and subclones. **a** Chromosome counts of Mol/MSM-1, -2, and -3 lines at p8. **b** Chromosome counts of gene trap clones obtained from Mol/MSM-1. Metaphase spreads of ES cells were prepared, and 50 metaphase spreads were counted for each cell line

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.

Injection of Mol/MSM-1 cells into B6 × BDF1 blastocysts was able to produce germline chimeras, whereas injection into ICR blastocysts resulted in only a low percentage of chimerism. When the ES cell line derived from the 129 strain was used for gene targeting, the efficiency of germline chimera production was believed to be higher when injected into C57BL/6 blastocysts compared with those from ICR mice (Nagy et al. 2003). This was the case for the ES cell line derived from the MSM/Ms strain, although we used B6 × BDF1 recipient embryos, instead of C57BL/6 embryos, in order to obtain a larger number of embryos. The difference in body size may also be related to the low level of chimera production with ICR blastocyst injection; MSM/Ms mice have a small body size (about 10 g at 8 weeks of age), which is almost one-third that of ICR mice. The different growth rate between MSM/Ms cells and ICR cells might prevent normal development of chimeras.

The use of genetically engineered mice is now indispensable for functional analysis of the mammalian genome. Phenotypes of knockout or knockin mice are profoundly influenced by their genetic backgrounds. Most laboratory mouse strains are derived from a few suppliers and have been selected for special traits, such as susceptibility to cancer. Therefore, the phenotypes observed in laboratory mouse strains might not reflect those of wild animals, including humans. Because wild-derived mouse strains, such as MSM/Ms, have not been selected for any particular traits, mutations in MSM/Ms mice could result in different phenotypes from those caused by the same mutations in common laboratory mouse strains, thus providing new insights into gene function. Thus, the Mol/MSM-1 ES line should provide an excellent new tool for the study of functional genomics.

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References

Abe K, Noguchi H, Tagawa K, Yuzuriha M, Toyoda A et al (2004) Contribution of Asian mouse subspecies *Mus musculus molossinus* to genomic constitution of strain C57BL/6J, as defined by BAC-end sequence-SNP analysis. Genome Res 14:2439–2447

Araki K, Imaizumi T, Sekimoto T, Yoshinobu K, Yoshimuta J et al (1999) Exchangeable gene trap using the Cre/mutated lox system. Cell Mol Biol (Noisy-le-grand) 45:737–750

Bonhomme F, Guénet JL (1996) The laboratory mouse and its wild relatives. In: Lyon MF, Rastan S, Brown SDM (eds) Genetic variants and strain of the laboratory mouse. Oxford University Press, Oxford, pp 1577–1596

Cheng J, Dutra A, Takesono A, Garrett-Beal L, Schwartzberg PL (2004) Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. Genesis 39:100–104

Fujiyama A, Watanabe H, Toyoda A, Taylor TD, Itoh T et al (2002) Construction and analysis of a human-chimpanzee comparative clone map. Science 295:131–134

Kobayashi M, Ohno T, Tsuchiya T, Horio F (2004) Characterization of diabetes-related traits in MSM and JF1 mice on high-fat diet. J Nutr Biochem 15:614–621

Koide T, Moriwaki K, Ikeda K, Niki H, Shiroishi T (2000) Multi-phenotype behavioral characterization of inbred strains derived from wild stocks of *Mus musculus*. Mamm Genome 11:664–670

Lawitts JA, Biggers JD (1993) Culture of preimplantation embryos. In: Wasserman PM, Depamphilis ML (eds) Methods in enzymology, vol 225. Academic Press, San Diego, CA, pp 153–164

Miyashita N, Moriwaki K (1987) H-2-controlled genetic susceptibility to pulmonary adenomas induced by urethane and 4-nitroquinoline 1-oxide in A/Wy congenic strains. Jpn J Cancer Res 78:494–498

Moriwaki K, Shiroishi T, Yonekawa H (1994) Genetics in wild mice. Japan Scientific Societies Press/Karger, Tokyo/Basel

Nagy A, Gocza E, Diaz EM, Prideaux VR, Ivanyi E et al (1990) Embryonic stem cells alone are able to support fetal development in the mouse. Development 110:815–821

Nagy A, Gertsenstein M, Vintersten K, Behringer R (2003) Manipulating the mouse embryo. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Nakanishi M, Tazawa H, Tsuchiya N, Sugimura T, Tanaka T et al (2007) Mouse strain differences in inflammatory responses of colonic mucosa induced by dextran sulfate sodium cause differential susceptibility to PhIP-induced large bowel carcinogenesis. Cancer Sci 98:1157–1163

Nakao K, Nakagata N, Katsuki M (1997) Simple and efficient vitrification procedure for cryopreservation of mouse embryos. Exp Anim 46:231–234

Okumoto M, Mori N, Miyashita N, Moriwaki K, Imai S et al (1995) Radiation-induced lymphomas in MSM, (BALB/cHeA × MSM) F1 and (BALB/cHeA × STS/A) F1 hybrid mice. Exp Anim 44:43–48

Peli J, Schmoll F, Laurincik J, Brem G, Schellander K (1996) Comparison of aggregation and injection techniques in producing chimeras with embryonic stem cells in mice. Theriogenology 45:833–842

Robertson EJ (1987) Embryo-derived stem cell lines. In: Robertson EJ (ed) Teratocarcinomas and embryonic stem cells. A practical approach. IRL Press, Oxford, pp 71–112

Shimizukawa R, Sakata A, Hirose M, Takahashi A, Iseki H et al (2005) Establishment of a new embryonic stem cell line derived from C57BL/6 mouse expressing EGFP ubiquitously. Genesis 42:47–52

Shinmen A, Honda A, Ohkawa M, Hirose M, Ogonuki N et al (2007) Efficient production of intersubspecific hybrid mice and embryonic stem cells by intracytoplasmic sperm injection. Mol Reprod Dev 74:1081–1088

Taniwaki T, Haruna K, Nakamura H, Sekimoto T, Oike Y et al (2005) Characterization of an exchangeable gene trap using pU-17 carrying a stop codon-beta geo cassette. Dev Growth Differ 47:163–172

Tokunaga T, Tsunoda Y (1992) Efficacious production of viable germ-line chimeras between embryonic stem (ES) cells and 8-cell stage embryos. Dev Growth Differ 34:561–566

Yonekawa H, Moriwaki K, Gotoh O, Miyashita N, Matsushima Y et al (1988) Hybrid origin of Japanese mice "*Mus musculus molossinus*": evidence from restriction analysis of mitochondrial DNA. Mol Biol Evol 5:63–78

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TECHNICAL REPORT

Enhanced expression of human cDNA by phosphoglycerate kinase promoter-puromycin cassette in the mouse transthyretin locus

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Abstract To produce a humanized mouse, it is critical to obtain a correct expression of a human gene/cDNA after insertion into a mouse locus. We previously generated a targeted allele in which the PGK-neo cassette, flanked by lox71 and loxP, was inserted into the first exon of the mouse endogenous transthyretin (Ttr) gene in ES cells. Using these ES cells, we showed that a human transthyretin (TTR) cDNA with the PGK-puro cassette can be efficiently inserted into this locus by Cre-mediated recombination, and that the human TTR cDNA was expressed in a tissue-specific manner under the control of the mouse endogenous Ttr promoter. To examine whether the PGK-puro cassette or IRES could affect the expression of human TTR cDNA, we generated four mouse lines using Cre and Flp-mediated recombination. The mouse line containing the PGK-puro cassette, but not IRES, exhibited quantitatively and temporally similar expression of human TTR cDNA. Removal of the PGK-puro cassette significantly downregulated the expression of the cDNA. The insertion of IRES sequence upstream of the human TTR cDNA resulted in decreased expression, even in the presence of the PGK-puro cassette. The mouse line containing IRES, but not PGK-puro, showed the lowest level of expression. These results suggest that the PGK-puro cassette is necessary to obtain the enhanced expression of a co-existing human cDNA in the mouse Ttr locus, even though the expression of co-existing cDNA was under the control of the mouse endogenous promoter.

Keywords Phosphoglycerate kinase promoter · Internal ribosomal entry site · Humanized mouse · Cre-mediated recombination · Transthyretin

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Introduction

Gene targeting has been very useful for the study of gene function in mice and for the production of mouse models of human disease. However, a human

genetic disease can be caused by a variety of mutations of the gene, and phenotypes are variable from one mutation to another. For example, the autosomal dominant disease, familial amyloidotic polyneuropathy (FAP), can be caused by many different mutations in the transthyretin (TTR) gene. So far, 110 TTR variants have been identified and more than 90 of these are associated with human amyloidosis. In case of a substitution of valine with methionine at position 30 (Val30Met), a patient shows mild symptoms, while the Leu55Pro mutation shows very aggressive phenotypes (Jacobson et al. 1992). Thus, we need to develop various mouse lines with different human TTR gene variants to analyze their roles in pathological conditions. Although a high throughput method for generating targeting vector using 96 well plates have been developed recently (Chan et al. 2007), it is still laborious to make each targeting vector and select targeted ES clones.

We previously developed an exchangeable gene targeting method (Wang et al. 2008; Zhao et al. 2008) based on the exchangeable gene trap method (Araki et al. 1999, 2002; Taniwaki et al. 2005). Using this method, we successfully produced a null targeted allele at the mouse transthyretin (Ttr) gene locus as the first step, followed by highly efficient replacement of the phosphoglycerine kinase (PGK)-puro cassette with a human TTR gene, using the Cre-loxP system (Zhao et al. 2008). In this system, lox71 and loxP sites in the mouse targeted allele were recombined with lox66 and loxP sites in the replacement vector, resulting in the insertion of a human cDNA into the mouse TTR locus. Thus, we can produce many kinds of a humanized mouse with a mutation of interest using this exchangeable gene targeting method. In these experiments, it is critical to express the human gene in qualitatively and quantitatively similar manners as the mouse endogenous gene. Thus, the present study was undertaken to examine the effect of PGK promoter or the internal ribosomal entry site (IRES) on the expression of the co-introduced human TTR cDNA. We generated four replacement alleles containing the human TTR cDNA with or without the PGK-puro cassette or IRES sequence. The results showed that the PGK-puro cassette enhanced transcription of human TTR cDNA, but that the IRES sequence suppressed transcription of the human TTR cDNA.

Materials and methods

Plasmid construction

Production of ES cells with the targeted allele which contains lox71-PGK-neo-loxP-polyA cassette at the mouse Ttr locus, was performed according to previously described methods (Zhao et al. 2008). The first step was the production of the targeted null allele with the simultaneous introduction of lox71 and loxP sites, by homologous recombination (Fig. 1a–c). To replace the PGK-neo gene with a human TTR cDNA in the initial targeted embryonic stem (ES) cells, two types of replacement vectors were produced (Fig. 1d). One of the vectors contained a rabbit β-globin intron II-human TTR cDNA-polyA-FRT-PGK-puro-FRT cassette flanked by lox66 and loxP sites (Fig. 1d left). Using this vector, the second replacement vector was constructed by insertion of an IRES element from the encephalomyocarditis virus (ECMV) (Jang and Wimmer 1990) into the 5′ region of the human TTR cDNA (Fig. 1d right).

ES cell culture and production of targeted ES clone

Standard procedures were used for TT2 ES cell culture (Niwa et al. 1993). TT2 ES cells were electroporated (Bio-Rad Gene Pulser at 800 V, 3.0 μF) with 30 μg of linearized targeting vector to produce the original targeted ES clone with the null mutation in the mouse Ttr gene locus. Colony selection with G418 (250 μg/ml, Sigma, USA) was started 24 h after electroporation and continued for 7 days. Single colonies were isolated and genomic DNA was prepared for genotyping.

Production of four replacement alleles containing human TTR cDNA

The targeted ES clones were co-electroporated (Bio-Rad Gene Pulser at 400 V, 125 μF) with 20 μg replacement vector and 20 μg pCAGGS-Cre plasmid (Araki et al. 1997), resulting in the production of the replaced alleles, I(−)P(+) and I(+)P(+) (where I = IRES and P = PGK-puro; Fig. 1e). Here, puromycin was used for positive selection. The presence of replacement alleles was confirmed by Southern blot and PCR analyses. The mouse lines with replaced

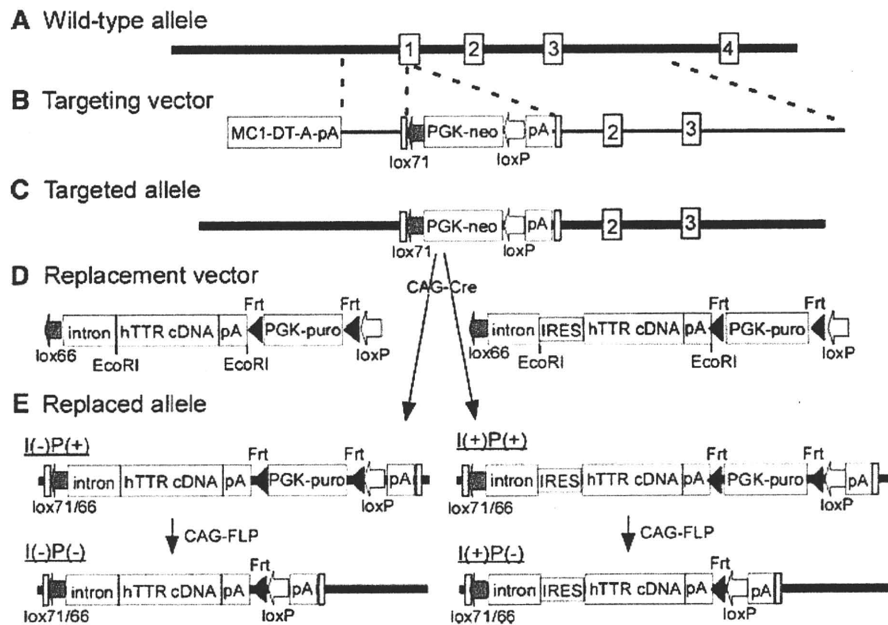

Fig. 1 Creation of the four replaced alleles for human TTR cDNA. **a–c** The targeted null allele containing lox71 and loxP sites was produced by homologous recombination in the first step. **d** Construction of replacement vectors. We constructed the first replacement vector, which contains the human TTR cDNA and PGK-puro. This vector was then used to construct the second replacement vector by the insertion of an IRES in front of the human TTR cDNA. **e** Production of replaced alleles. The replaced alleles, I(−)P(+) and I(+)P(+), were created by Cre-mediated recombination using the replacement vectors. The replaced alleles, I(−)P(−) and I(+)P(−), were created by removal of PGK-puro cassette through Flp-mediated site-specific recombination

alleles, I(−)P(+) and I(+)P(+), were subsequently mated with CAGGS-Flp transgenic mice (Taniwaki et al. 2005) to remove the PGK-puro cassette, resulting in the production of two other replaced alleles, I(−)P(−) and I(+)P(−) (Fig. 1e).

Generation of mice with a series of replaced alleles

Chimeric mice were produced by aggregation of ES cells with eight-cell embryos from ICR mice. Chimeric embryos were then transferred into pseudopregnant recipients. Chimeric male mice were backcrossed to C57BL/6 J females (Nippon Clea, Kanagawa, Japan) and mice from the fifth generation onwards were used in subsequent experiments.

PCR and Southern blot analyses for genotyping

The genotypes of ES cells and mice were determined by PCR analyses and confirmed by Southern blot analysis, using genomic DNA from ES cells or tails. In PCR analysis, a primer set, A9-s (5′-CGTAGAGC GAGTGTTCCG-3′) and SP-A (5′-CAGTGTATA TCATTGTAACC-3′), was used to detect the replaced allele. A primer set, IRES2 (5′-ACCACGGGG ACGTGGTTTTC-3′) and hT-r1 (5′-TGTCATCAG CAGCCTTTC-3′), was used to detect the presence of the IRES sequence. A primer set, hT-F2 (5′-CAC CAATCCCAAGGAATGAG-3′) and PGK-r2 (5′- TA AAGCGCATGCTCCAGAC-3′), was used to confirm the excision of PGK-puro cassette. For Southern blot analysis, 10 μg DNA from cells or mice with the replaced allele were digested with *Bgl*II. The digested DNAs were electrophoresed on a 1.2%-agarose gel and blotted onto nylon membranes (Hybond-N+, Amersham, Tokyo, Japan). After the membranes were cross-linked by exposure to ultraviolet light (UV Stratalinker 1800, Stratagene, La Jolla, CA), hybridization was performed using a puro-specific probe (see Fig. 2c) prepared by a DIG DNA labeling and detection kit (Roche, Tokyo, Japan).

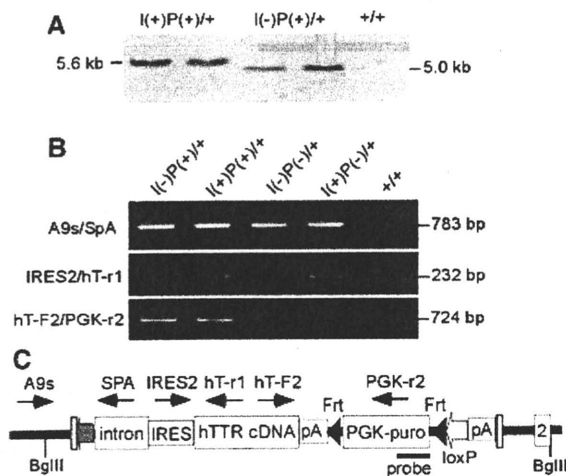

Fig. 2 Genotyping of replaced ES clones and mice. **a** Southern blot analysis. The size of bands from I(+)P(+)/+ (5.6 kb) mice was greater than that from I(−)P(+)/+ (5 kb) mice when the genomic DNAs were digested with *Bgl*II and hybridized with a puro-specific probe. **b** Genotyping. All replaced alleles gave 783-bp bands when amplified by A9-s/SP-A primer set. The replaced alleles with the IRES sequence gave 232-bp bands when amplified by IRES2/hT-r1 primer set. The replaced alleles with the PGK-puro cassette gave 724-bp bands when amplified by the hT-F2/PGK-r2 primer set. **c** PCR primers, puro-specific probe and *Bgl*II digestion sites in the replaced allele

Northern blot analysis

For Northern blot analysis, total RNAs isolated from livers and brains were electrophoresed on a 1.2%-denaturing formaldehyde/MOPS-containing agarose gel and transferred to nylon membranes (Hybond-N+; Amersham, Tokyo, Japan). After the RNAs were cross-linked by exposure to UV light, the membrane was prehybridized and then hybridized using a human or mouse TTR cDNA-specific RNA probe, or the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) RNA probe prepared by DIG RNA labeling and detection kit (Roche, Tokyo, Japan). To analyze the temporal expression of the human TTR cDNA, RNAs were extracted from the livers and brains of fetuses at embryonic day 15.5 (E15.5), newborn, and 1-, 2-, 3-, 4-, 8-, and 12-week-old mice.

Western blot analysis

Livers were homogenized in lysate buffer (HEPES 50 mmol/l, pH 7.4, NaCl 150 mmol/l, Triton X-100 0.1%, glycerol 10%, NaF 1 mmol/l, sodium orthovanadate 2 mmol/l, ethylenediaminetetraacetic acid 1 mmol/l, and protease inhibitor cocktail (1:100 dilution; Sigma–Aldrich, Tokyo, Japan). The extracts (200 μg of protein per lane) and mouse serum (6 μl of mouse serum diluted 1:6 in 0.9% NaCl per lane) were applied to 15% polyacrylamide gels, electrophoresed, and transferred to an immobilon polyvinylidene difluoride filter (Millipore, Billerica, MA, USA). Primary antibodies were used at the indicated dilutions: rabbit anti-human TTR antibody (diluted 1:1,000; Abcam, Cambridge, UK), rabbit anti-bovine serum albumin (BSA) antibody (diluted 1:1,000; Upstate, USA), and rabbit anti-β-actin antibody (diluted 1:1,000; Sigma–Aldrich, Tokyo, Japan). An anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (Amersham Biosciences Corp, Piscataway, NJ, USA) was used for detection. The intensity of the bands was quantified by densitometry using ImageJ software (version 1.40, a program inspired by NIH image; http://rsb.info.nih.gov/ij/docs/index.html).

Semiquantitative RT-PCR analysis

Total RNA from tissues was isolated using the acid guanidium thiocyanate phenol-chloroform extraction procedure. RNA samples from various tissues were reverse-transcribed according to the manufacturer's instructions for Thermoscript™ RT-PCR system (Invitrogen Life Technologies, Tokyo, Japan). About 82% of nucleotide sequence is identical between the mouse and human TTR coding region. Thus, we first examined whether we could use a set of primers with the same sequence for detection of both mouse and human TTR cDNA. We found that the following primers could be used for such purpose. Sequences were as follows: sense primer (mhTTR-F1), 5′-GTCCTCTGATGGTCAAAGT-3′ and antisense primer (mhTTR-R2), 5′-GAGTCGTTGGCTGTGAA-3′. The primer set was expected to give PCR products of 271 bp. PCR consisted of an initial denaturation cycle at 94°C for 5 min, followed by the 27 cycles at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 30 s. An additional cycle at 72°C for 7 min completed the amplification process. The primers used for detection of Gapdh mRNA were as follows: sense primer, 5′-TGC TGA GTA TGT CGT GGA GTC-3′ and antisense primer, 5′-AGA AGG GGT GGA GAT GAT GAC-3′. The primer set

for Gapdh was expected to give PCR products of 103 bp. PCR consisted of an initial denaturation cycle at 94°C for 5 min, followed by 23 cycles consisting of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 30 s. An additional cycle at 72°C for 7 min completed the amplification process. Amplified PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Construction of CAG-hTTR and CAG-IRES-hTTR

EcoRI fragment containing either human TTR cDNA-pA or IRES-human TTR cDNA-pA was excised from the replacement vector (see Fig. 1d) and inserted into *Eco*RI site of pCAGGS (Niwa et al. 1991) to produce CAG-hTTR or CAG-IRES-hTTR, respectively.

BMT10 cell culture and transfection

BMT10 cells were grown in DMEM medium (Sigma–Aldrich, Tokyo, Japan) with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Thermo Scientific, Waltham, MA, USA) and maintained at 37°C in 5% CO_2 atmosphere.

BMT10 cells were grown to confluence in 10-cm dishes and subcultured into 6-cm dishes with 3×10^5 cells/dish for 24 h before co-transfection. The transfection procedure was performed with the Lipofectamine 2000 reagent (Invitrogen Life Technologies, Tokyo, Japan). In brief, 2 μg of CAG-hTTR and 2.2 μg of CAG-IRES-hTTR were diluted in 600 μl of Opti-MEM (Invitrogen Life Technologies, Tokyo, Japan). In another tube, 8.4 μl of Lipofectamine reagent were diluted into 600 μl of Opti-MEM. Then, the components of the two tubes were mixed and incubated for 20 min at room temperature. The above DNA-Lipofectamine complex was added to each dish. Five hours after co-transfection, medium was changed with DMEM containing 10% FBS. Co-transfection was carried out for 29 h.

Analysis of mRNA stability following inhibition of transcription by actinomycin D

Analysis on mRNA stability was carried out according to the method described by Wang et al. (Keetch et al. 2005) with some modification. Briefly, 29 h after transfection, the transcription inhibitor actinomycin D (20 μg/ml) (Sigma–Aldrich, Tokyo, Japan) was added into the cell culture. The cells were harvested at 0, 8, 16, 24, 32, 40 h after treatment with the actinomycin D. Total RNA of the cells was extracted and the hTTR and IRES-hTTR mRNA levels were determined by Northern blot analysis. The hTTR or IRES-hTTR mRNA levels normalized to 18S rRNA are shown as a function of actinomycin D treatment time.

Statistical analysis

In the present study, at least three independent experiments were carried out. Statistical analysis was performed applying unpaired Student's *t*-tests. The results are expressed as means ± SE. $P < 0.05$ was considered to be a statistically significant difference.

Results

Establishment of four different replacement alleles for human TTR cDNA

Production of the human TTR replaced alleles was done in three steps. The first step was the production of the targeted null allele with the simultaneous introduction of lox71 and loxP sites by homologous recombination (Fig. 1a–c). The second step was the introduction of the human TTR cDNA with or without an IRES sequence, to produce the replaced allele. The replacement vectors containing a rabbit *β*-globin intron-human TTR cDNA-polyA-FRT-PGK-puro-FRT cassette (Fig. 1d left) or a rabbit *β*-globin intron-IRES-human TTR cDNA-polyA-FRT-PGK-puro-FRT cassette (Fig. 1d right) were introduced to the targeted allele by Cre-mediated recombination between lox71/lox66 and loxP/loxP to produce the replaced alleles, I(−)P(+) and I(+)P(+) (Fig. 1e). The third step was the excision of PGK-puro by mating with CAGGGS-Flp transgenic mice to produce the replaced alleles, I(−)P(−) and I(+)P(−) (Fig. 1e). Thus, four human TTR alleles were produced in terms of the presence or absence of PGK-puro or IRES sequences. The first replaced allele, I(−)P(+), contained only the PGK-puro cassette. The second replaced allele,

I(−)P(−), contained neither IRES nor PGK-puro. The third replaced allele, I(+)P(+), contained both IRES and PGK-puro. The fourth replaced allele, I(+)P(−), contained IRES, but not PGK-puro (Fig. 1e).

Genotyping of ES cell clones and targeted mice

ES cell clones and mice were screened for targeted recombination by Southern blot and PCR analysis. First, we examined whether the replaced allele, I(−)P(+) or I(+)P(+), carried a single insertion of human TTR-PGK-puro or IRES-human TTR-PGK-puro, respectively by Southern blot analysis. As shown in Fig. 2a, the presence of bands of the predicted sizes indicated a single targeted insertion in the replaced alleles, I(−)P(+) and I(+)P(+). An IRES sequence was inserted in the I(+)P(+), therefore, the size of the bands from the I(+)P(+) mice (5.6 kb) was greater than that from the I(−)P(+) mice (5 kb), when the genomic DNAs were digested with *BglII* and hybridized using puro-specific probe (Fig. 2a, c). The 783-bp PCR products obtained using the A9-s/Sp-A primer set indicated the presence of β-globin intron-human TTR cDNA. The 232-bp PCR products obtained using the IRES2/hT-r1 primer set indicated the presence of IRES in the replaced allele. The 724-bp PCR products obtained using the hT-F-2/PGK-r2 primer set indicated the presence of PGK-puro cassette in the replaced allele (Fig. 2b, c).

Human TTR mRNA levels in the liver

Northern blot analysis was carried out to detect human TTR mRNAs in the liver using human TTR cDNA-specific RNA probe (Fig. 3a). We detected the expected sizes of mRNAs, 1,242, 1,830, 1,242, or 1,830 bp, for alleles I(−)P(+), I(+)P(+), I(−)P(−), or I(+)P(−), respectively (Fig. 3a, b). As shown in Fig. 3a, the level of human TTR mRNA was the highest in I(−)p(+) mice. The effect of PGK-puro cassette can be assessed by comparing the levels of mRNA expression between I(−)P(+) and I(−)P(−) mice. Presence of the PGK-puro cassette in I(−)P(+) mice resulted in 3 times increase of the mRNA levels of human TTR than that in I(−)p(−) mice. On the other hand, the effect of IRES can be assessed by comparing the levels of mRNA expression between the I(−)P(+) and the I(+)P(+) mice. Addition of IRES in I(+)P(+) mice, resulted in a decrease in the mRNA levels of human TTR to one third of that in I(−)p(+) mice. Furthermore, the level of mRNA expression in I(+)P(+) was similar to that in I(+)P(−), suggesting that the IRES counteracted the increased transcription by the PGK-puro cassette. The mRNA level in I(−)P(−) was low and similar to that in I(+)P(−), suggesting that the mRNA level is already low in the absence of PGK-puro cassette. The mRNA levels of human TTR in homozygous mice were higher than those in heterozygous mice in all strains.

Levels of TTR protein in the serum

As shown in Fig. 4, serum levels of TTR protein in heterozygous mice of the four strains are roughly proportional to those of mRNAs in the liver. However, serum levels of the human TTR protein in homozygous mice were lower than those in heterozygous mice. This was due to inefficient binding of human TTR tetramers to mouse retinol binding protein, which leads to dissociation of TTR tetramers into monomers and loss of TTR monomers from the circulation through glomerular filtration (Kato et al. 1984; Zhao et al. 2008). The rabbit anti-human TTR antibody (Abcam, Cambridge, UK) used in this experiment was quite specific for human TTR, as the intensity of mouse serum TTR band in wild-type mouse serum was very weak (Fig. 4).

Spatial, quantitative and temporal expression of human TTR in I(−)P(+)/I(−)P(+)

We previously reported that the tissue-specificity of human TTR cDNA in I(−)P(+)/+ mice was exactly the same as that of the endogenous mouse Ttr gene in Northern blot analysis (Zhao et al. 2008). We also examined the tissue-specificity of human TTR cDNA in other strains. We detected mRNAs for human TTR slightly in the liver and brain, but not in other tissues. To compare the spatial expression of human TTR mRNA with that of the mouse Ttr more in detail, semiquantitative RT-PCR analyses were carried out using the primers common to the human TTR and the mouse Ttr. The human TTR cDNA was expressed in brain, eyes, liver, kidney and intestine, the tissues where the mouse Ttr gene was also expressed (Fig. 5a). It is of interest that human TTR cDNA was expressed in the intestine in which the human TTR expression was not observed in human (see EST

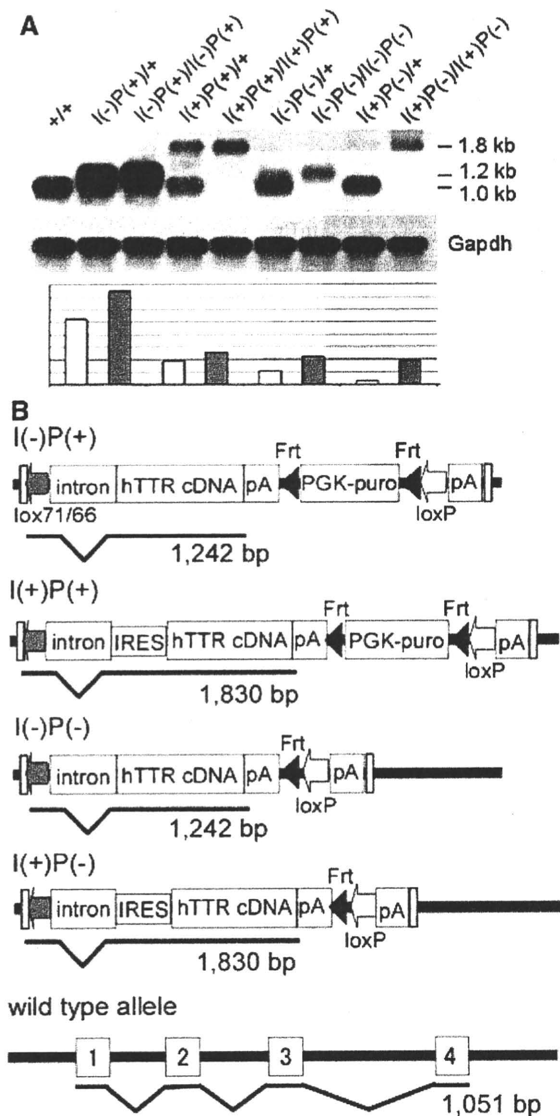

Fig. 3 Human TTR mRNA level in the liver. **a** Northern blot analysis. The human TTR mRNA level in the liver was analyzed using a human TTR cDNA-specific RNA probe. I(−)P(+) mice showed the highest level of expression. Insertion of an IRES or removal of the PGK-puro cassettes resulted in significant decrease in transcription and hence, the lowest level of expression. The levels of human TTR mRNA in homozygous mice were higher than those in heterozygous mice. **b** The transcription of human or mouse TTR mRNA and expected sizes of mRNA from each replaced allele

profile in NCBI Unigene database: http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.2108).p://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.2108).

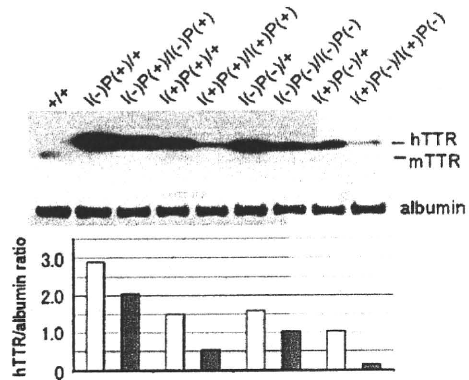

Fig. 4 TTR protein levels in the serum. Serum TTR protein level was determined by western blot analysis. The level of human TTR protein was higher in the sera of I(−)P(+) mice than those in the sera of other mice. Serum levels of human TTR protein in homozygous mice were lower than those in heterozygous mice. This was due to the instability of the human TTR homotetramers (Zhao et al. 2008). As the rabbit anti-human TTR antibody (Abcam, Cambridge, UK) is quite specific to human TTR protein, mouse TTR protein was barely detectable in this Western blot analysis

To compare the level of human TTR mRNA expression with that of the mouse Ttr, semiquantitative RT-PCR analyses were carried out using the primers common to the human TTR and the mouse Ttr. The level of human TTR mRNA in the livers of I(−)P(+)/I(−)P(+) mice was similar to that of mouse Ttr mRNA in control mice, while the levels of human TTR mRNA in brain and eyes of I(−)P(+)/I(−)P(+) mice were lower than those of mouse Ttr mRNA (Fig. 5b).

To analyze the temporal expression of the human TTR, RNAs were extracted from the livers and brains of fetuses at embryonic day 15.5 (E15.5), newborn, and 1-, 2-, 3-, 4-, 8- and 12-week-old I(−)P(+)/+ mice. In the liver and brain, the expression levels of both human TTR and mouse Ttr mRNA increased gradually up to 12 weeks of age (Fig. 5c), with both mRNAs showing a similar pattern of expression.

Analysis of mRNA stability

The mRNA stability was compared between CAG-hTTR and CAG-IRES-hTTR constructs. The levels of mRNA transcripts at 0, 8, 16, 24, 32, and 40 h after treatment with actinomycin D were determined by Northern blot analysis. Both mRNAs were quite

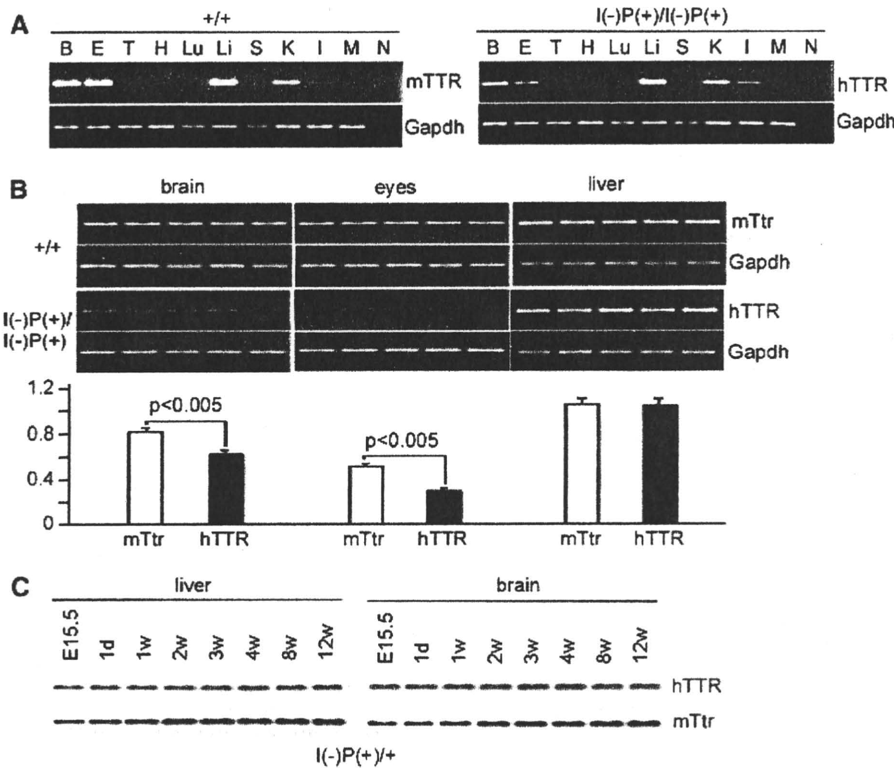

Fig. 5 Spatial, quantitative and temporal expression of human TTR cDNA. **a** Spatial expression of mouse and human TTR. The human TTR was expressed in the same tissues as the mouse Ttr. *B* brain, *E* eyes, *T* thymus, *H* heart, *Lu* lung, *Li* liver, *S* spleen, *K* kidney, *I* intestine, *M* skeletal muscle, *N* no RNA. **b** Quantitative expression of human and mouse TTR in brain, eye and liver. mRNA levels were quantified by densitometric analysis. *: $P < 0.05$ ($n = 5$). The expression levels of human TTR in the liver of I(−)P(+)/I(−)P(+) mice were similar to those of mouse Ttr in control mice (+/+). **c** Temporal expression of the human and mouse TTR gene. Northern blot analysis was performed using RNAs obtained from the liver and brain of I(−)P(+)/+ mice

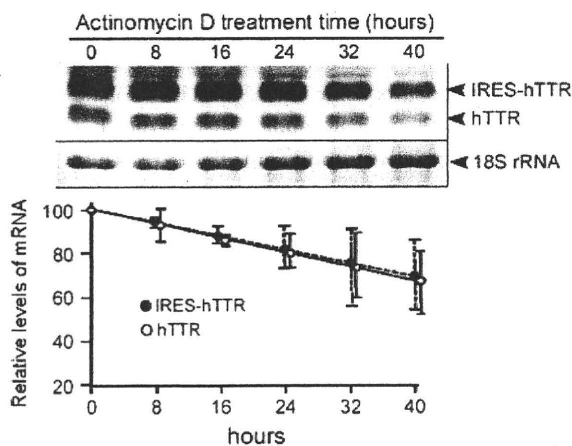

Fig. 6 Analysis of mRNA stability. hTTR or IRES-hTTR mRNA levels were determined relative to 18S rRNA by Northern blot analysis. Each time point was normalized to time 0. Stability of IRES-hTTR was similar to that of hTTR mRNA stable and there were no significant differences in stability between hTTR and IRES-hTTR mRNAs (Fig. 6), suggesting that the presence of IRES did not affect the stability of mRNA.

Discussion

In this study, we generated four mouse lines with different replaced alleles using Cre and Flp-mediated recombination. We showed that the PGK-puro cassette enhanced the expression of co-existing human TTR cDNA, but that the IRES sequence suppressed transcription of human TTR cDNA.

The PGK-neo cassette is widely used as a selectable marker for homologous recombination in ES cells. The PGK-neo cassette, which was retained in

the targeted allele, can function as a strong promoter or enhancer for neighboring genes (Johnson and Friedmann 1990; Nesterova et al. 2003; Scacheri et al. 2001; Zhu et al. 2007). For example, Zhu et al. (2007) demonstrated that the insertion of a PGK-neo cassette into the proximal upstream region of Zic3 gene caused an increase in Zic3 transcription but not an altered pattern of expression. Seidl et al. (1999) reported position-dependent inhibition of class-switch recombination by PGK-neo cassettes inserted into the immunoglobulin heavy chain constant locus. Germline transcription of $C\gamma3$ gene located upstream of PGK-neo cassette was impaired, while germline expression of $C\gamma2A$ and $C\varepsilon$ genes located downstream of was not impaired. Sun and Storb (2001) also showed that insertion of PGK-neo upstream of the $J\lambda1$ region dramatically increased $V\lambda1$-$J\lambda1$ recombination and $J\lambda1$ germline transcription levels in pre-B cells and thymus cells, apparently due to the strong housekeeping PGK promoter driving the neo gene. These results suggest that the PGK promoter can function as a promoter/enhancer or through a position effect for neighboring genes. In this manuscript, the PGK promoter, appeared to behave similarly, resulting in quantitatively and temporally similar expression of co-introduced human cDNA. We previously reported that the tissue-specificity of human TTR cDNA was exactly the same as that of the endogenous mouse Ttr gene. We (Wang et al. 2008) also created a $Spink3^{+/lacZ}$ knock-in mouse, in which lacZ with the PGK-puro was inserted into the Spink3 locus and analyzed the spatiotemporal expression profile of Spink3, using in situ hybridization (ISH) and X-gal staining. We demonstrated that expression patterns of lacZ were very similar to those of ISH, suggesting that $Spink3^{lacZ}$ was expressed correctly under the control of the promoter/enhancer of the endogenous Spink3 gene. Alternatively, we may be able to use another enhancer such as a recombination enhancer, but not transcriptional enhancer, to drive the correct expression of introduced human cDNA, because the insertion of a PGK-puro may have different effects when inserted into another gene locus. We need further investigation to analyze whether it is desirable to have a selectable marker cassette in the other genetic locus or to find an enhancer suitable for the correct expression of human cDNA in any gene locus. In any case, the PGK-puro cassette maintains the quantitatively, temporally, and spatially similar expression of the co-introduced cDNA in the mouse Ttr locus, even though the expression of co-existing cDNA was under the control of the mouse endogenous promoter.

In 1988, Pelletier and Sonenberg (1988) as well as Jang et al. (1988) demonstrated that IRES, originally found in eukaryotic viruses, initiates ribosome binding and translation in a cap-independent manner. Since then, bicistronic vectors in which the first gene is translated in a cap-dependent manner and the second one in an IRES-dependent manner have been widely used for in vitro and in vivo applications, from cultured cells and transgenic animals to gene therapy (for a review see Hellen and Sarnow 2001). Thus, we also examined whether an IRES element could enhance translation of human TTR cDNA. Surprisingly, the insertion of an IRES element decreased both the transcription and translation of human TTR cDNA. Mizuguchi et al. (2000) demonstrated that the level of IRES-dependent second gene expression in a bicistronic vector ranged from 6 to 100% of the cap-dependent first gene expression in several cultured cell lines and in mouse liver in vivo. Hasegawa et al. (2007) also reported similar results using human ES cells. In both cases, they observed the effect of IRES on translation, but not on transcription, in a single-promoter-driven multicistronic protein expression cassette. In contrast, we observed downregulation of transcription, although the stability of IRES-hTTR was similar to that of hTTR. In our case, the cap site is located in the mouse endogenous gene locus. Although the reason for the low mRNA expression is still unknown, it is possible that an IRES present in the vicinity of a mouse endogenous promoter exerts a distance effect on consecutive transcription units, resulting in the low level transcription of human TTR cDNA.

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References

Araki K, Araki M, Yamamura K (1997) Targeted integration of DNA using mutant lox sites in embryonic stem cells. Nucleic Acids Res 25:868–872

Araki K, Imaizumi T, Sekimoto T, Yoshinobu K, Yoshimuta J, Akizuki M, Miura K, Araki M, Yamamura K (1999) Exchangeable gene trap using the Cre/mutated lox system. Cell Mol Biol (Noisy-le-grand) 45:737–750

Araki K, Araki M, Yamamura K (2002) Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites. Nucleic Acids Res 30:e103

Chan W, Costantino N, Li R, Lee SC, Su Q, Melvin D, Court DL, Liu P (2007) A recombineering based approach for high-throughput conditional knockout targeting vector construction. Nucleic Acids Res 35:e64

Hasegawa K, Cowan AB, Nakatsuji N, Suemori H (2007) Efficient multicistronic expression of a transgene in human embryonic stem cells. Stem Cells 25:1707–1712

Hellen CU, Sarnow P (2001) Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev 15:1593–1612

Jacobson DR, McFarlin DE, Kane I, Buxbaum JN (1992) Transthyretin Pro55, a variant associated with early-onset, aggressive, diffuse amyloidosis with cardiac and neurologic involvement. Hum Genet 89:353–356

Jang SK, Wimmer E (1990) Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev 4:1560–1572

Jang SK, Krausslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer E (1988) A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J Virol 62:2636–2643

Johnson P, Friedmann T (1990) Limited bidirectional activity of two housekeeping gene promoters: human HPRT and PGK. Gene 88:207–213

Kato M, Kato K, Goodman DS (1984) Immunocytochemical studies on the localization of plasma and of cellular retinol-binding proteins and of transthyretin (prealbumin) in rat liver and kidney. J Cell Biol 98:1696–1704

Keetch CA, Bromley EH, McCammon MG, Wang N, Christodoulou J, Robinson CV (2005) L55P transthyretin accelerates subunit exchange and leads to rapid formation of hybrid tetramers. J Biol Chem 280:41667–41674

Mizuguchi H, Xu Z, Ishii-Watabe A, Uchida E, Hayakawa T (2000) IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. Mol Ther 1:376–382

Nesterova TB, Johnston CM, Appanah R, Newall AE, Godwin J, Alexiou M, Brockdorff N (2003) Skewing X chromosome choice by modulating sense transcription across the Xist locus. Genes Dev 17:2177–2190

Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199

Niwa H, Araki K, Kimura S, Taniguchi S, Wakasugi S, Yamamura K (1993) An efficient gene-trap method using poly A trap vectors and characterization of gene-trap events. J Biochem 113:343–349

Pelletier J, Sonenberg N (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 334:320–325

Scacheri PC, Crabtree JS, Novotny EA, Garrett-Beal L, Chen A, Edgemon KA, Marx SJ, Spiegel AM, Chandrasekharappa SC, Collins FS (2001) Bidirectional transcriptional activity of PGK-neomycin and unexpected embryonic lethality in heterozygote chimeric knockout mice. Genesis 30:259–263

Seidl KJ, Manis JP, Bottaro A, Zhang J, Davidson L, Kisselgof A, Oettgen H, Alt FW (1999) Position-dependent inhibition of class-switch recombination by PGK-neor cassettes inserted into the immunoglobulin heavy chain constant region locus. Proc Natl Acad Sci USA 96:3000–3005

Sun T, Storb U (2001) Insertion of phosphoglycerine kinase (PGK)-neo 5′ of Jlambda1 dramatically enhances VJlambda1 rearrangement. J Exp Med 193:699–712

Taniwaki T, Haruna K, Nakamura H, Sekimoto T, Oike Y, Imaizumi T, Saito F, Muta M, Soejima Y, Utoh A, Nakagata N, Araki M, Yamamura K, Araki K (2005) Characterization of an exchangeable gene trap using pU-17 carrying a stop codon-beta geo cassette. Dev Growth Differ 47:163–172

Wang J, Ohmuraya M, Hirota M, Baba H, Araki K, Yamamura K (2008) Expression pattern of the serine protease inhibitor Kazal Type 3 (Spink3) gene during the mouse embryonic development. Histochem Cell Biol 130:387–397

Zhao G, Li Z, Araki K, Haruna K, Yamaguchi K, Araki M, Takeya M, Ando Y, Yamamura KI (2008) Inconsistency between hepatic expression and serum concentration of transthyretin in mice humanized at the transthyretin locus. Genes Cells 13:1257–1268

Zhu L, Peng JL, Harutyunyan KG, Garcia MD, Justice MJ, Belmont JW (2007) Craniofacial, skeletal, and cardiac defects associated with altered embryonic murine Zic3 expression following targeted insertion of a PGK-NEO cassette. Front Biosci 12:1680–1690

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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.