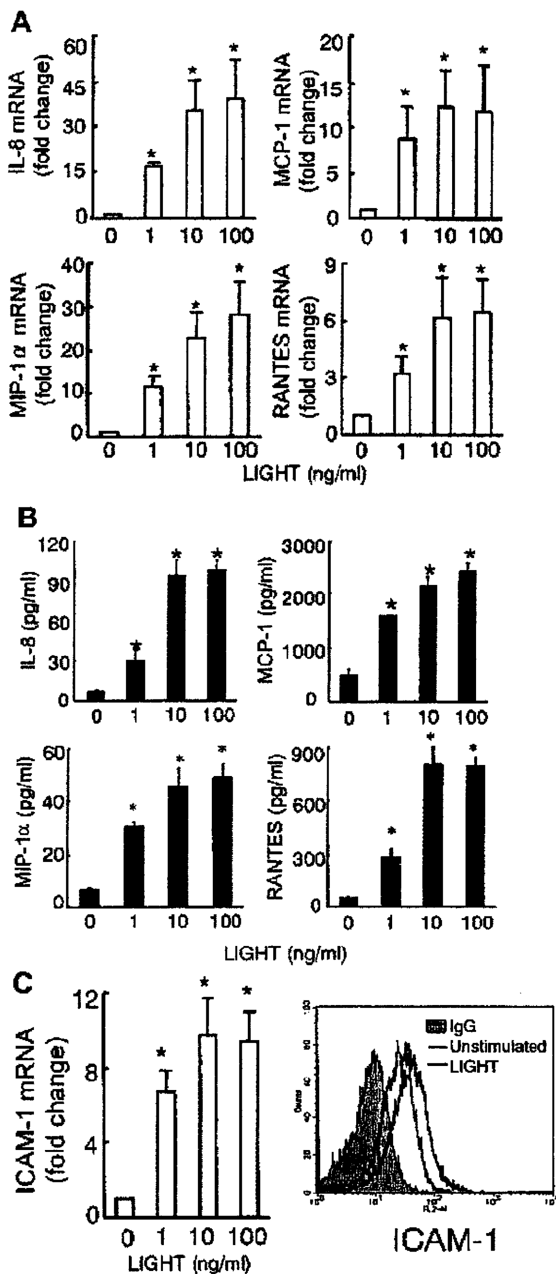

Figure 4. Upregulation of IL-8, MCP-1, MIP-1α, RANTES, and ICAM-1 expression in RA-FLS by LIGHT. A. RA-FLS were stimulated with the indicated concentrations of LIGHT for 3 h, and real-time quantitative PCR was performed to determine levels of IL-8, MCP-1, MIP-1α, and RANTES mRNA expression. Values are shown as means ± SD per fold change compared with controls. B. RA-FLS were stimulated with the indicated concentrations of LIGHT for 72 h. Concentrations of IL-8, MCP-1, MIP-1α, and RANTES in cell culture supernatants were determined by multiplex bead array assays. Values are shown as means ± SD pg/ml. C. RA-FLS were stimulated with the indicated concentrations of LIGHT for 3 h, and real-time quantitative PCR was performed to determine levels of ICAM-1 mRNA expression. ICAM-1 surface expression on RA-FLS was detected by flow cytometry after stimulation with 10 ng/ml LIGHT for 24 h. All analyses were carried out on 4 RA-FLS lines; flow cytometry profiles of one representative result are shown. $^*p < 0.05$.

ed with LIGHT (Figure 4C). Similar increases in VCAM-1 mRNA and protein expression were also seen when stimulated with LIGHT (data not shown). Moreover, we investigated whether knockdown of HVEM or LTßR suppressed this series of LIGHT-induced gene expression in RA-FLS. Compared with control siRNA, LTßR siRNA, but not HVEM siRNA, significantly decreased the expression of IL-8, MCP-1, and ICAM-1 mRNA induced by LIGHT (Figure 5). Similarly, LTßR siRNA decreased the LIGHT-induced expression of IL-1ß, IL-6, GM-CSF, RANTES, and MIP-1α mRNA in FLS (data not shown).

Activation of NF-κB in RA-FLS via LTβR by LIGHT. It is known that activation of NF-κB has a key role in inflammatory disease[23]. Several studies have shown that LIGHT activates the transcription factor NF-κB in different cell types[7,9,13,24-26]. To investigate the involvement of NF-κB in LIGHT-induced gene expression, we examined the effect of the NF-κB inhibitor PDTC on the expression of IL-8, MCP-1, and ICAM-1 by real-time quantitative PCR. PDTC completely abolished the LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 (Figure 6A). The LIGHT-induced expression of IL-1ß, IL-6, GM-CSF, RANTES, and MIP-1α mRNA in RA-FLS was also inhibited by PDTC treatment (data not shown). The concentration of PDTC used in these experiments had no cytotoxic effect, as demonstrated by cell viability studies using trypan blue exclusion, which showed that > 95% of cells remained viable over the entire period of the experiment (data not shown).

In the immunocytofluorescence analysis using anti-NF-κB p65 mAb, enhanced nuclear translocation of NF-κB p65 was observed in LIGHT-stimulated RA-FLS (Figure 6B). Further, Western blotting using anti-IκBa mAb showed that IκBa degradation was induced by LIGHT, and that IκBa degradation was inhibited by LTßR siRNA, but not by HVEM siRNA (Figure 6C).

DISCUSSION

We observed that LIGHT, but not HVEM or LTßR, is overexpressed in the synovial tissues of patients with RA compared with those of patients with OA. The expression of LIGHT was not detected in RA-FLS, which comprise one of the major components of the RA synovium. RA synovium is histologically characterized by prominent infiltration of macrophages and lymphocytes[27]. Although LIGHT has been supposed to be produced by activated T lymphocytes *in vitro*[4,5], a recent study reported that LIGHT was overexpressed in CD68-positive macrophages in RA synovial tissue compared with those in OA synovial tissue, and that expression levels of LIGHT were low in areas rich in lymphocytes[19]. Thus, macrophages rather than FLS and lymphocytes could be the major source of LIGHT in the RA synovium.

We further demonstrated that *in vitro*-cultured RA-FLS express HVEM and LTßR, which implies that RA-FLS are

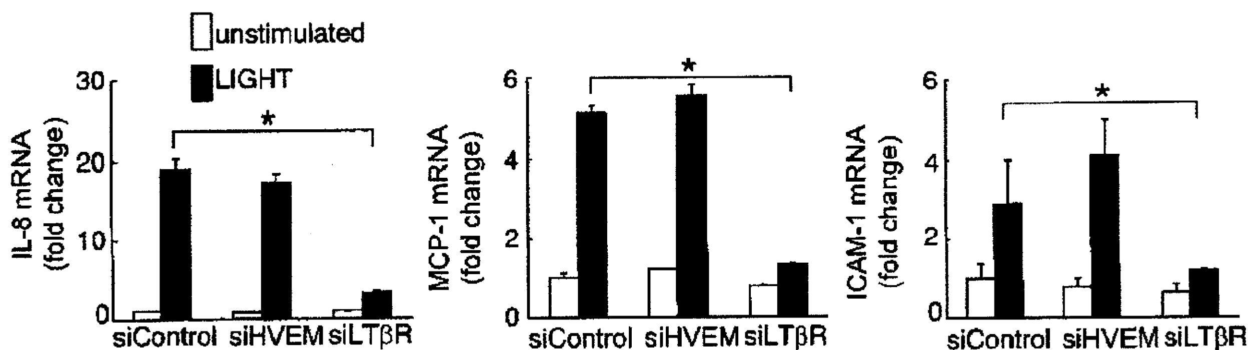

Figure 5. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 in RA-FLS via LTßR. RA-FLS were transfected with control, HVEM, or LTßR siRNA using Lipofectamine 2000. After 96 h incubation, cells were stimulated with 10 ng/ml LIGHT for an additional 3 h. Levels of IL-8, MCP-1, and ICAM-1 mRNA were analyzed by real-time quantitative PCR. Values are shown as means ± SD per fold change compared with controls. All analyses were carried out on 4 RA-FLS lines. $*p < 0.05$.

target cells of LIGHT. Indeed, we first showed that LIGHT had a stronger RA-FLS growth-promoting activity than PDGF, in lower concentrations. The proliferation of RA-FLS is one of the most critical pathological changes in RA. Thus, our findings suggest that increased expression of LIGHT might lead to the synovial hyperplasia of RA. Anticytokine therapies targeting TNF-α, IL-1ß, and IL-6 have been used to treat patients with RA, and it has been demonstrated that such treatments may suppress the accompanying bone destruction as well as the synovitis[28,29]. In addition, recent studies have indicated that LIGHT reduces Fas-mediated apoptosis in FLS[30], that LIGHT may function as a mediator of bone resorption through the induction of osteoclastogenesis[31], and that LTßR-Ig protein blocks the induction of experimental arthritis in mice[18]. Thus, a neutralizing antibody against LIGHT could be a useful tool for inhibition of synovial hyperplasia and bone destruction in RA.

The enhanced effects of LIGHT on RA-FLS proliferation were significantly inhibited by LTßR siRNA, but not by HVEM siRNA, suggesting that LTßR, rather than HVEM, is involved in the LIGHT-induced proliferation of RA-FLS. The exact mechanism by which LIGHT influences RA-FLS proliferation through LTßR is unknown. A potential mechanism underlying RA-FLS proliferation induced by LIGHT may involve cell-cycle regulators, including cyclin-dependent kinases (CDK). The mammal cell cycle is controlled by holoenzymes composed of a catalytic CDK and regulatory cyclin. The expression level of p21 was reduced in RA synovial linings and FLS compared with the level in patients with OA[32]. Overexpression of p21 or p16 by adenoviral-mediated delivery suppresses FLS growth *in vitro*[33,34]. Further, LIGHT induces cell proliferation, downregulates the CDK inhibitors p21, p27 and p53, and inversely upregulates cyclin D and Rb hyperphosphorylation in vascular smooth muscle cells[13]. Thus, it is possible that LIGHT promotes FLS proliferation by shortening the cell cycle of FLS in RA. Wang, *et al* reported that LTßR-null mice show reduced BrdU incorporation in dendritic cells[35]. This supports our claim that LTßR signaling is involved in the proliferation of RA-FLS.

We observed that LIGHT also induces the production of inflammatory cytokines and chemokines and expression of adhesion molecules on RA-FLS. Inflammatory cytokines and chemokines induce the migration of cells and release of mediators of inflammation and angiogenesis, and could be involved in the pathogenesis of RA[1,2,36]. The increased expression of ICAM-1 and VCAM-1 adhesion molecules on activated endothelial cells enhances the recruitment of monocytes, lymphocytes, and neutrophils, leading to inflammation. These findings indicate that LIGHT might play an important role in inflammation in the synovial lining layer, as well as in its hyperplasia. A recent study revealed that LIGHT upregulates the expression of ICAM-1, VCAM-1, and IL-6 in RA-FLS via NF-κB activation[30,37]. Although these reports are consistent with our present results, it has not been clear which of 2 receptors is involved in the induction of these genes in FLS. Our knockdown analysis using siRNA revealed that LIGHT induces proliferation and gene expression by signaling via LTßR, but not HVEM. Braun, *et al* have shown that LTßR is expressed on RA-FLS, and that LTa1ß2, a ligand for LTßR, induces expression of inflammatory cytokines, chemokines, and ICAM-1[38]. This supports our claim that LTßR signaling is involved in the activation of RA-FLS. The NF-κB transcription factor is certainly involved in cytokine- and chemokine-driven responses and is a point of convergence for several upstream proinflammatory pathways[23]. Indeed, NF-κB activation appears to be an important factor in RA, as the expression of NF-κB is enhanced in lining cells[39,40] and in the cartilage-pannus junction in the RA synovium[41]. In our study, treatment with PDTC blocked LIGHT-induced IL-8, MCP-1, and ICAM-1 expression, suggesting that the effects of LIGHT are mediated through NF-κB. The involvement of NF-κB in LIGHT-induced proinflammatory responses was further confirmed

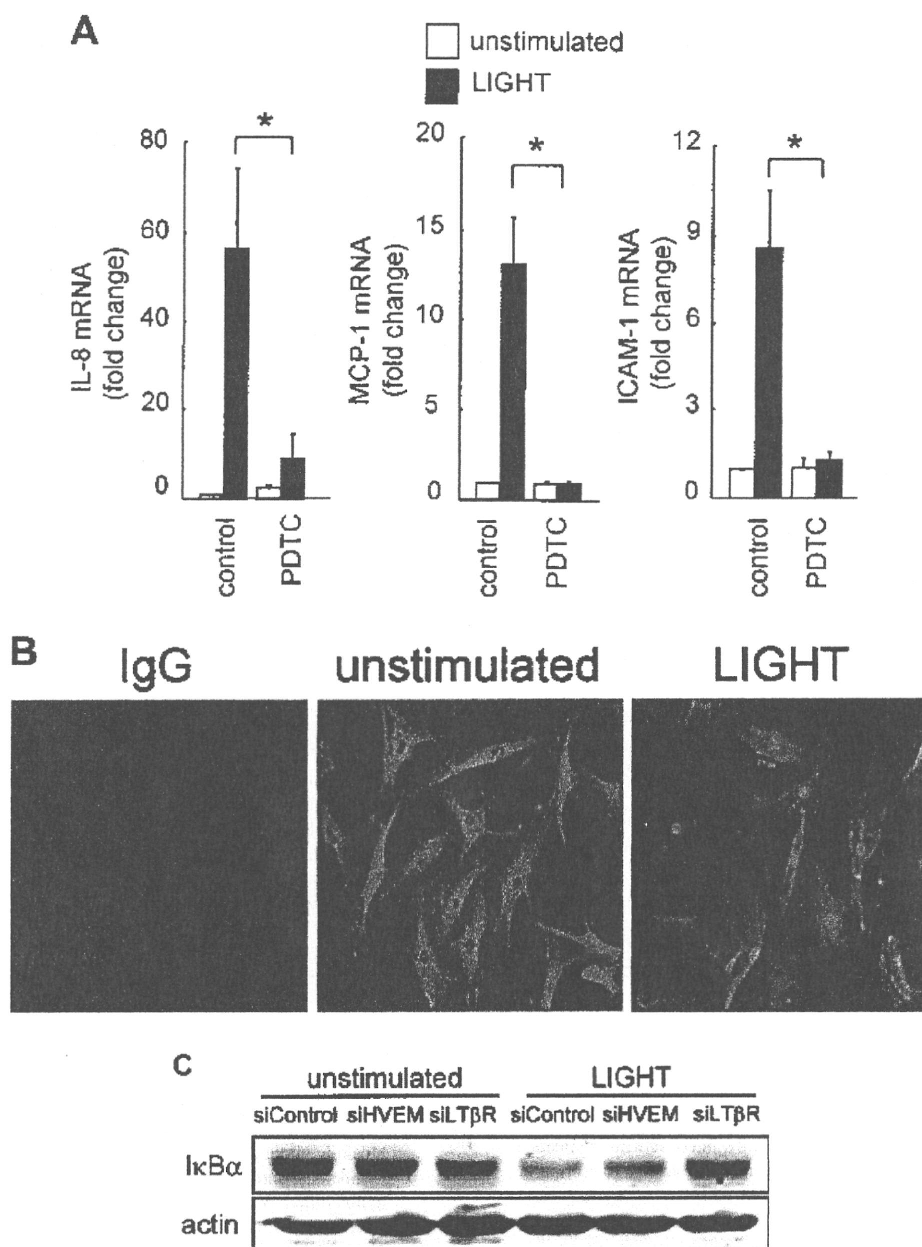

Figure 6. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 through NF-κB-mediated pathways. A. FLS were stimulated with 10 ng/ml LIGHT for 3 h with or without preincubation for 30 min with 30 μM PDTC. Levels of IL-8, MCP-1, and ICAM-1 mRNA were analyzed by real-time quantitative PCR. Values are shown as means ± SD per fold change compared with control. All analyses were carried out on 4 RA-FLS lines. *$p < 0.05$. B. Immunofluorescence staining for NF-κB p65 in RA-FLS. Control in which primary antibodies were replaced with control IgG (left panel); unstimulated RA-FLS (middle); and RA-FLS stimulated with 10 ng/ml LIGHT for 30 min (right). Results are representative of 2 experiments using 2 FLS lines. C. 96 h after siRNA transfection, cells were stimulated with 10 ng/ml LIGHT for 40 min. IκBa degradation was analyzed by immunoblotting. Results are representative of 2 experiments using 2 RA-FLS lines.

by the LIGHT-induced nuclear translocation of NF-κB p65. Moreover, LIGHT induced IκBa degradation in RA-FLS, an effect that was inhibited by LTßR siRNA, but not by HVEM siRNA. These findings are consistent with studies showing that LTßR ligation can lead to activation of NF-κB[24,42-45]. However, it is unknown why LIGHT prefers the LTßR signaling pathway in RA-FLS, even though HVEM is also expressed on these cells.

We have demonstrated that LIGHT is overexpressed in RA synovial tissues and SF. LIGHT induced increased production of inflammatory cytokines, chemokines, and adhesion molecules through NF-κB activation, as well as proliferation of RA-FLS. These findings indicate that LIGHT signaling via LTßR plays an important role in the pathogenesis of RA by affecting key processes such as the proliferation and activation of RA-FLS. Therefore, regulation of LIGHT-LTßR signaling may represent a new therapeutic target for the treatment of RA.

REFERENCES

1. Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 2001;344:907-16.
2. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol 1996;14:397-440.
3. Gitter BD, Labus JM, Lees SL, Scheetz ME. Characteristics of human synovial fibroblast activation by IL-1 beta and TNF alpha. Immunology 1989;66:196-200.
4. Mauri DN, Ebner R, Montgomery RI, et al. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. Immunity 1998;8:21-30.
5. Morel Y, Schiano de Colella JM, Harrop J, et al. Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. J Immunol 2000;165:4397-404.
6. Zhai Y, Guo R, Hsu TL, et al. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. J Clin Invest 1998;102:1142-51.
7. Tamada K, Shimozaki K, Chapoval AI, et al. LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. J Immunol 2000;164:4105-10.
8. Kwon BS, Tan KB, Ni J, et al. A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. J Biol Chem 1997;272:14272-6.
9. Zou GM, Hu WY. LIGHT regulates CD86 expression on dendritic cells through NF-kappaB, but not JNK/AP-1 signal transduction pathway. J Cell Physiol 2005;205:437-43.
10. Marsters SA, Ayres TM, Skubatch M, Gray CL, Rothe M, Ashkenazi A. Herpesvirus entry mediator, a member of the tumor necrosis factor receptor (TNFR) family, interacts with members of the TNFR-associated factor family and activates the transcription factors NF-kappaB and AP-1. J Biol Chem 1997;272:14029-32.
11. Force WR, Walter BN, Hession C, et al. Mouse lymphotoxin-beta receptor. Molecular genetics, ligand binding, and expression. J Immunol 1995;155:5280-8.
12. Harrop JA, McDonnell PC, Brigham-Burke M, et al. Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. J Biol Chem 1998;273:27548-56.
13. Wei CY, Chou YH, Ho FM, Hsieh SL, Lin WW. Signaling pathways of LIGHT induced macrophage migration and vascular smooth muscle cell proliferation. J Cell Physiol 2006;209:735-43.
14. Wang J, Lo JC, Foster A, et al. The regulation of T cell homeostasis and autoimmunity by T cell-derived LIGHT. J Clin Invest 2001;108:1771-80.
15. Scholz H, Sandberg W, Damas JK, et al. Enhanced plasma levels of LIGHT in unstable angina: possible pathogenic role in foam cell formation and thrombosis. Circulation 2005;112:2121-9.
16. Chang YH, Hsieh SL, Chao Y, Chou YC, Lin WW. Proinflammatory effects of LIGHT through HVEM and LTbetaR interactions in cultured human umbilical vein endothelial cells. J Biomed Sci 2005;12:363-75.
17. Otterdal K, Smith C, Oie E, et al. Platelet-derived LIGHT induces inflammatory responses in endothelial cells and monocytes. Blood 2006;108:928-35.
18. Fava RA, Notidis E, Hunt J, et al. A role for the lymphotoxin/LIGHT axis in the pathogenesis of murine collagen-induced arthritis. J Immunol 2003;171:115-26.
19. Kim WJ, Kang YJ, Koh EM, Ahn KS, Cha HS, Lee WH. LIGHT is involved in the pathogenesis of rheumatoid arthritis by inducing the expression of pro-inflammatory cytokines and MMP-9 in macrophages. Immunology 2005;114:272-9.
20. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
21. Takeuchi E, Tomita T, Toyosaki-Maeda T, et al. Establishment and characterization of nurse cell-like stromal cell lines from synovial tissues of patients with rheumatoid arthritis. Arthritis Rheum 1999;42:221-8.
22. Butler DM, Leizer T, Hamilton JA. Stimulation of human synovial fibroblast DNA synthesis by platelet-derived growth factor and fibroblast growth factor. Differences to the activation by IL-1. J Immunol 1989;142:3098-103.
23. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest 2001;107:7-11.
24. Kim YS, Nedospasov SA, Liu ZG. TRAF2 plays a key, nonredundant role in LIGHT-lymphotoxin beta receptor signaling. Mol Cell Biol 2005;25:2130-7.
25. Hikichi Y, Matsui H, Tsuji I, et al. LIGHT, a member of the TNF superfamily, induces morphological changes and delays proliferation in the human rhabdomyosarcoma cell line RD. Biochem Biophys Res Commun 2001;289:670-7.
26. Matsui H, Hikichi Y, Tsuji I, Yamada T, Shintani Y. LIGHT, a member of the tumor necrosis factor ligand superfamily, prevents tumor necrosis factor-alpha-mediated human primary hepatocyte apoptosis, but not Fas-mediated apoptosis. J Biol Chem 2002;277:50054-61.
27. Tak PP, Bresnihan B. The pathogenesis and prevention of joint damage in rheumatoid arthritis: advances from synovial biopsy and tissue analysis. Arthritis Rheum 2000;43:2619-33.
28. Elliott MJ, Maini RN, Feldmann M, et al. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. Arthritis Rheum 1993;36:1681-90.
29. Choy EH, Isenberg DA, Garrood T, et al. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum 2002;46:3143-50.
30. Pierer M, Brentano F, Rethage J, et al. The TNF superfamily member LIGHT contributes to survival and activation of synovial fibroblasts in rheumatoid arthritis. Rheumatology Oxford 2007;46:1063-70.
31. Edwards JR, Sun SG, Locklin R, et al. LIGHT (TNFSF14), a novel mediator of bone resorption, is elevated in rheumatoid arthritis. Arthritis Rheum 2006;54:1451-62.
32. Perlman H, Bradley K, Liu H, et al. IL-6 and matrix metalloproteinase-1 are regulated by the cyclin-dependent kinase inhibitor p21 in synovial fibroblasts. J Immunol 2003;170:838-45.
33. Nonomura Y, Kohsaka H, Nasu K, Terada Y, Ikeda M, Miyasaka N. Suppression of arthritis by forced expression of cyclin-dependent kinase inhibitor p21(Cip1) gene into the joints. Int Immunol 2001;13:723-31.
34. Taniguchi K, Kohsaka H, Inoue N, et al. Induction of the

p16INK4a senescence gene as a new therapeutic strategy for the treatment of rheumatoid arthritis. Nat Med 1999;5:760-7.
35. Wang YG, Kim KD, Wang J, Yu P, Fu YX. Stimulating lymphotoxin beta receptor on the dendritic cells is critical for their homeostasis and expansion. J Immunol 2005;175:6997-7002.
36. Moser B, Willimann K. Chemokines: role in inflammation and immune surveillance. Ann Rheum Dis 2004;63:ii84-ii89.
37. Kang YM, Kim SY, Kang JH, et al. LIGHT up-regulated on B lymphocytes and monocytes in rheumatoid arthritis mediates cellular adhesion and metalloproteinase production by synoviocytes. Arthritis Rheum 2007;56:1106-17.
38. Braun A, Takemura S, Vallejo AN, Goronzy JJ, Weyand CM. Lymphotoxin beta-mediated stimulation of synoviocytes in rheumatoid arthritis. Arthritis Rheum 2004;50:2140-50.
39. Marok R, Winyard PG, Coumbe A, et al. Activation of the transcription factor nuclear factor-kappa B in human inflamed synovial tissue. Arthritis Rheum 1996;39:583-91.
40. Asahara H, Asanuma M, Ogawa N, Nishibayashi S, Inoue H. High DNA-binding activity of transcription factor NF-kappa B in synovial membranes of patients with rheumatoid arthritis. Biochem Mol Biol Int 1995;37:827-32.
41. Benito MJ, Murphy E, Murphy EP, van den Berg WB, FitzGerald O, Bresnihan B. Increased synovial tissue NF-kappa B1 expression at sites adjacent to the cartilage-pannus junction in rheumatoid arthritis. Arthritis Rheum 2004;50:1781-7.
42. Mackay F, Majeau GR, Hochman PS, Browning JL. Lymphotoxin beta receptor triggering induces activation of the nuclear factor kappa B transcription factor in some cell types. J Biol Chem 1996;271:24934-8.
43. Nakano H, Oshima H, Chung W, et al. TRAF5, an activator of NF-kappa B and putative signal transducer for the lymphotoxin-beta receptor. J Biol Chem 1996;271:14661-4.
44. Van Arsdale TL, Van Arsdale SL, Force WR, et al. Lymphotoxin-beta receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor kappa B. Proc Natl Acad Sci USA 1997;94:2460-5.
45. Chang YH, Hsieh SL, Chen MC, Lin WW. Lymphotoxin beta receptor induces interleukin 8 gene expression via NF-kappa B and AP-1 activation. Exp Cell Res 2002;278:166-74.

Araki et al. BMC Biotechnology 2010, 10:29
http://www.biomedcentral.com/1472-6750/10/29

BMC Biotechnology

METHODOLOGY ARTICLE

Open Access

Comparative analysis of right element mutant *lox* sites on recombination efficiency in embryonic stem cells

Kimi Araki*, Yuka Okada, Masatake Araki and Ken-ichi Yamamura

Abstract

Background: Cre-mediated site-specific integrative recombination in mouse embryonic stem (ES) cells is a useful tool for genome engineering, allowing precise and repeated site-specific integration. To promote the integrative reaction, a left element/right element (LE/RE) mutant strategy using a pair of *lox* sites with mutations in the LE or RE of the *lox* sequence has previously been developed. Recombination between LE and RE mutant *lox* produces a wild-type *lox*P site as well as an LE+RE double mutant *lox* site, which has mutations in both sides and less affinity to Cre, resulting in stable integration. We previously demonstrated successful integrative recombination using *lox*71 (an LE mutant) and *lox*66 (an RE mutant) in ES cells. Recently, other LE/RE mutant *lox* sites showing higher recombination efficiency in *Escherichia coli* have been reported. However, their recombination efficiency in mammalian cells remains to be analyzed.

Results: Using ES cells, we compared six RE mutant *lox* sites, focusing on their recombination efficiency with *lox*71. All of the RE mutant *lox* sites showed similar recombination efficiency. We then analyzed the stability of the recombined product, i.e., the LE+RE double mutant *lox* site, under continuous and strong Cre activity in ES cells. Two RE mutants, *lox*JTZ17 and *lox*KR3, produced more stable LE+RE double mutant *lox* than did the *lox*66/71 double mutant.

Conclusion: The two mutant RE *lox* sites, *lox*JTZ17 and *lox*KR3, are more suitable than *lox*66 for Cre-mediated integration or inversion in ES cells.

Background

The bacteriophage P1-derived Cre/*lox* recombination system has been extensively used to engineer the genome of experimental animals [1,2]. Cre recombinase recognizes a 34-bp element, termed *lox*P, which is composed of two 13-bp inverted repeats that serve as Cre binding sites, and an 8-bp spacer region that participates in strand exchange during recombination (Figure 1a) [3,4]. Depending on the relative orientation of the *lox* sites with respect to one another, the recombination reaction can result in excision, inversion, or integration.

Integrative recombination is useful for the production of transgenic animals or cells because any DNA of interest can be introduced into a chromosomally located *lox* site. However, integrative recombination between wild-type *lox*P sites is inefficient due to re-excision through intramolecular recombination [5]. Studies of mutated *lox*P sites have revealed that two classes of mutations can promote Cre-mediated insertion or replacement. One class consists of heterospecific *lox* sites carrying mutation(s) in the central 8-bp spacer region [6-8]. Recombination does not occur between two *lox* sites differing in the spacer region, whereas *lox* sites with identical spacer regions can be recombined efficiently. Recombination using heterospecific *lox* sites is termed "recombinase mediated cassette exchange (RMCE)" [9], in which one chromosomally preinserted DNA cassette flanked by two different heterospecific *lox* sites is exchanged for another cassette on a targeting plasmid flanked by the same kind of heterospecific *lox* sites. To date, *lox*511 [10], *lox*2272 [11], and *lox*5171 [12] have been successfully used in embryonic stem (ES) cells.

The other class is the left element/right element (LE/RE) mutant strategy using LE mutant *lox* carrying muta-

* Correspondence: arakimi@gpo.kumamoto-u.ac.jp
[1] Institute of Resource Development and Analysis, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan
Full list of author information is available at the end of the article

© 2010 Araki et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

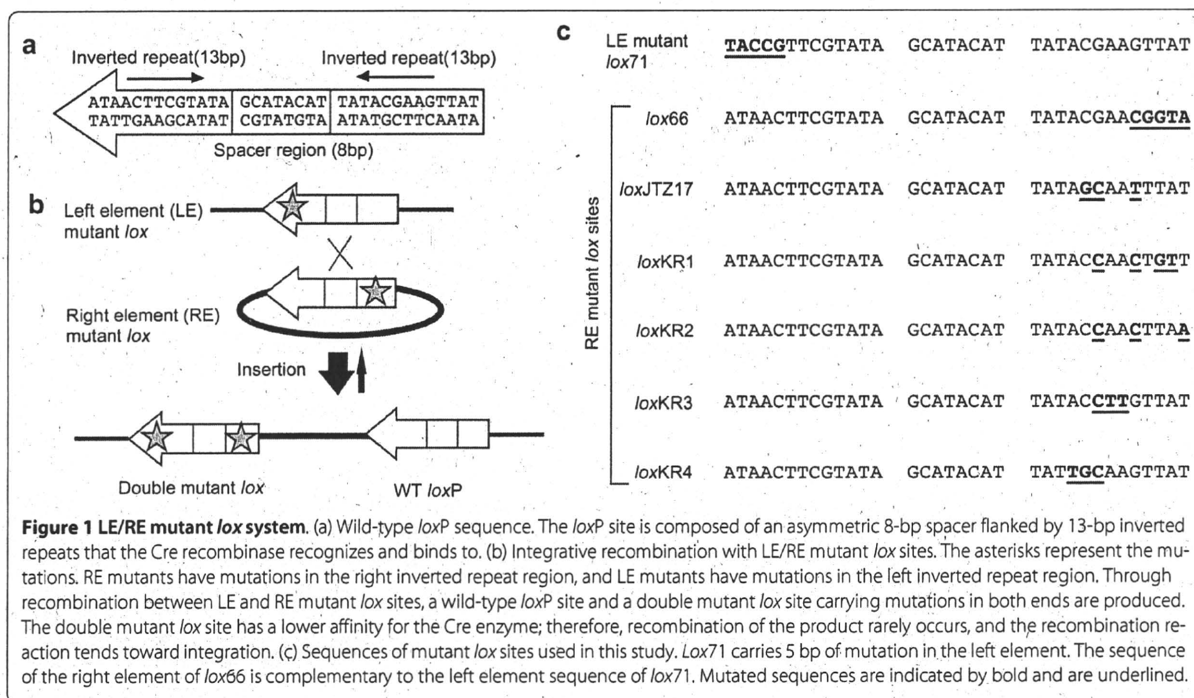

Figure 1 LE/RE mutant *lox* system. (a) Wild-type *loxP* sequence. The *loxP* site is composed of an asymmetric 8-bp spacer flanked by 13-bp inverted repeats that the Cre recombinase recognizes and binds to. (b) Integrative recombination with LE/RE mutant *lox* sites. The asterisks represent the mutations. RE mutants have mutations in the right inverted repeat region, and LE mutants have mutations in the left inverted repeat region. Through recombination between LE and RE mutant *lox* sites, a wild-type *loxP* site and a double mutant *lox* site carrying mutations in both ends are produced. The double mutant *lox* site has a lower affinity for the Cre enzyme; therefore, recombination of the product rarely occurs, and the recombination reaction tends toward integration. (c) Sequences of mutant *lox* sites used in this study. *Lox71* carries 5 bp of mutation in the left element. The sequence of the right element of *lox66* is complementary to the left element sequence of *lox71*. Mutated sequences are indicated by bold and are underlined.

tions in the left-inverted repeat region and RE mutant *lox* carrying mutations in the right-inverted repeat region [13]. Recombination between an LE mutant *lox* and an RE mutant *lox* results in the generation of a double mutant *lox* site having mutations in both ends and a wild-type *loxP* site. The double mutant *lox* site is not an effective substrate for Cre recombinase; therefore, the recombination reaction proceeds exclusively in one direction (Figure 1b). We have previously demonstrated successful integrative recombination using *lox71* (an LE mutant) and *lox66* (an RE mutant) in ES cells [14]. Moreover, two other groups have used *lox71/66* to induce unidirectional Cre-mediated inversion [15,16].

Although the integrative recombination efficiency using *lox* 71/66 is lower than RMCE efficiency using *loxP* and *lox2272* [17], the advantage of the LE/RE mutant *lox* strategy is its simplicity. Only one LE or RE *lox* site is required as a target for integrative recombination. Recently, Thomson et al. performed mutational analysis of LE/RE mutant *lox* sites using *Escherichia coli* and identified two novel LE/RE mutant *lox*, *lox*JT15 and *lox*JTZ17, which showed approximately 1500-fold higher integration rates than *lox71* and *lox66* [18]. If these novel mutant *lox* sites could also improve integration efficiency in ES cells, they would be useful tools for Cre-mediated integration in mammalian genomes.

One application of the Cre/mutant *lox* integration system is gene trapping in ES cells. Our group, as well as two other groups (Database for Exchangeable Gene Trap Clones, Sanger Institute Gene Trap Resources and Bay Genomics), have constructed gene trap vectors incorporating a *lox71* site and have generated over 20,000 gene trap cell lines, which are available to the academic community through the International Gene Trap Consortium database http://www.genetrap.org/index.html. With these trap clones, any DNA of interest can be inserted into a *lox71* site and expressed under the control of the trapped gene promoter [19]. Therefore, in this study, we focused on screening for efficient RE mutant *lox* possessing better recombination efficiency with the *lox71* site. Thomson et al. reported that the recombination efficiency between *lox*JTZ17 and *lox71* was 10 times higher than that between *lox66* and *lox71*. Although the 10-fold promoting effect is less than the 1500-fold effect obtained with *lox*JT15 and *lox*JTZ17, this improvement is considered sufficient because the recombination efficiency of *lox66* and *lox71* in ES cells is 2-16% [14]. Here, we used six RE mutant *lox* sites, including *lox66*, *lox*JTZ, and four newly synthesized RE mutant *lox* sites(*lox*KR1-4), and compared both their recombination efficiency and the stability of recombined products in ES cells.

Results and Discussion

Mutant RE lox sites

We synthesized four new RE *lox* sites (*lox*KR1-4) as well as *lox*JTZ17 (Figure 1c). *Lox*KR1 had four nucleotide

mutations; of these, three mutated nucleotides were the same as the mutations in *lox*66, and one nucleotide was the same as the mutation in *lox*JTZ17. *Lox*KR2-4 were designed to have three mutations, as did *lox*JTZ17, but in different positions.

Assessment of integrative recombination efficiency

To test whether these RE *lox* sites could promote recombination efficiency in ES cells, we used the same strategy as described previously [14](Figure 2a). For the chromosomal target *lox*71 site, the CAG promoter-*lox*71-bsr-pA was introduced into ES cells. We isolated four ES clones—Bs1, Bs17, Bs19, and Bs21—that carried a single copy of the chromosomal target construct. Integration vectors comprised an RE *lox* site on the 5' side of the promoterless *NLSlacZ* gene and a selection marker gene, MC1-*neo*-pA. The ES clones were coelectroporated with the integration vector and the Cre-expression vector pCAGGS-Cre (in their circular forms) and then selected with G418. The *cre* gene was transiently expressed, mediating site-specific recombination between the chromosomal *lox*71 and the RE *lox* on the integration plasmid; this resulted in site-specific integration. The neo cassette is active regardless of the integration site; therefore, both random integrants and site-specific recombinants become drug resistant. However, in the present study, only the colonies where site-specific integration had occurred were stained blue with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) because the *NLSLacZ* gene was inserted downstream of the CAG promoter through Cre-mediated recombination. As shown in Figure 2b and 2c, most of the blue colonies were uniformly blue, indicating that recombination occurred in the early stage of colony formation. The percentage of blue colonies represented the frequency of site-specific integration. When only the integration vector was electroporated, no blue colonies appeared (Figure 2d).

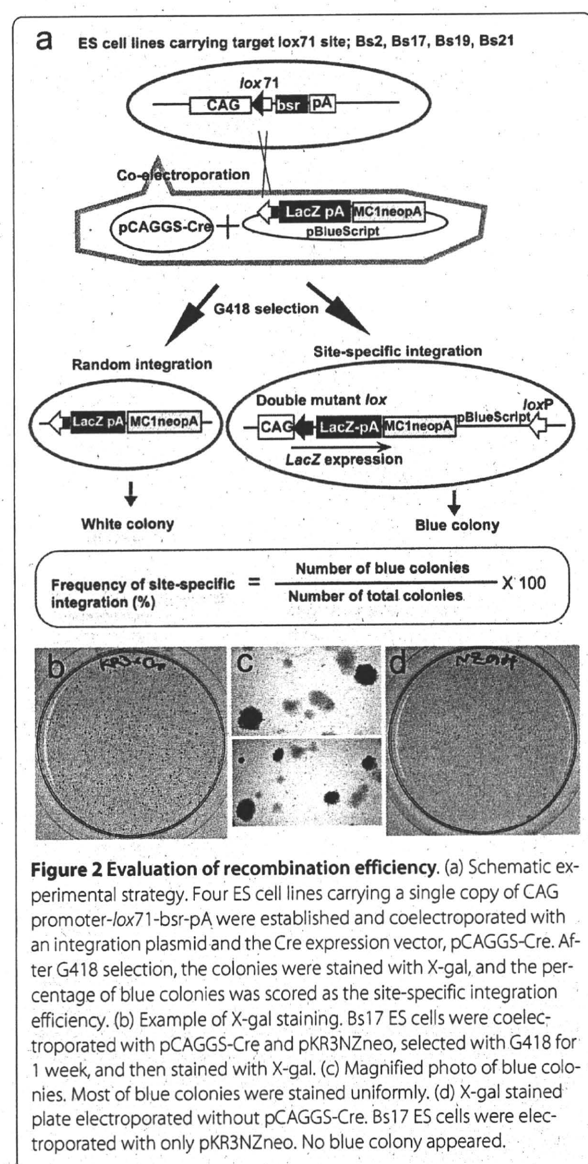

Figure 2 Evaluation of recombination efficiency. (a) Schematic experimental strategy. Four ES cell lines carrying a single copy of CAG promoter-*lox*71-bsr-pA were established and coelectroporated with an integration plasmid and the Cre expression vector, pCAGGS-Cre. After G418 selection, the colonies were stained with X-gal, and the percentage of blue colonies was scored as the site-specific integration efficiency. (b) Example of X-gal staining. Bs17 ES cells were coelectroporated with pCAGGS-Cre and pKR3NZneo, selected with G418 for 1 week, and then stained with X-gal. (c) Magnified photo of blue colonies. Most of blue colonies were stained uniformly. (d) X-gal stained plate electroporated without pCAGGS-Cre. Bs17 ES cells were electroporated with only pKR3NZneo. No blue colony appeared.

Site-specific integration frequencies

We constructed six integration vectors harboring different RE mutant *lox* and compared their recombination frequencies. Percentages of blue colonies, i.e., site-specific integration frequencies, in the four ES lines are shown in Table 1. The frequencies with the *lox*66-vector were 11-14%, and all other RE *lox* sites showed similar or slightly higher frequencies than were observed with *lox*66. Site-specific integration rates relative to the frequency with *lox*66 are shown in Figure 3. The differences observed were within a factor of 2, and there were no statistically significant differences among the RE *lox* sites (p = 0.37). Thus, the promoting effect of *lox*JTZ17 observed in *E. coli* was not clearly evident in ES cells. All new RE *lox* sites showed similar recombination efficiencies to *lox*JTZ17 and *lox*66.

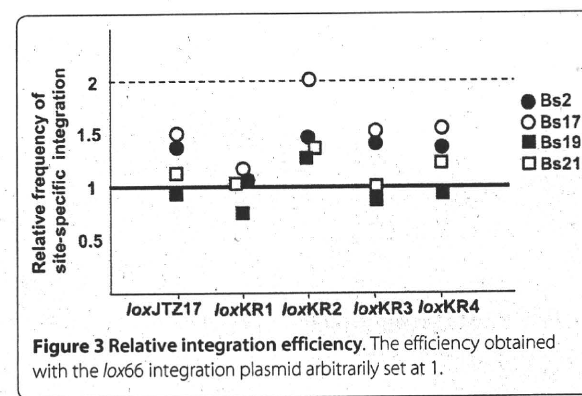

Figure 3 Relative integration efficiency. The efficiency obtained with the *lox*66 integration plasmid arbitrarily set at 1.

Table 1: Frequency of site-specific integration

Cell line	lox66	loxJTZ17	loxKR1	loxKR2	loxKR3	loxKR4
Bs2	13.4 ± 4.3	18.0 ± 5.4	13.9 ± 2.5	19.2 ± 6.9	18.0 ± 2.4	17.5 ± 1.2
Bs17	11.3 ± 0.6	17.2 ± 2.2	13.3 ± 1.5	22.7 ± 3.3	17.3 ± 2.4	17.5 ± 3.7
Bs19	14.2 ± 2.9	13.3 ± 3.1	10.5 ± 1.8	18.0 ± 5.7	11.9 ± 3.4	12.9 ± 1.1
Bs21	13.8 ± 1.3	15.8 ± 2.6	14.4 ± 2.3	18.9 ± 3.6	13.9 ± 4.5	16.9 ± 2.7

Twenty micrograms of the integration plasmid and 10 μg of the Cre-expressing vector were coelectroporated. After drug selection for 7 days, colonies were stained with X-gal, and the percentage of positive colonies was scored as the frequency of site-specific integration. The means ± SD of three independent electroporations are represented.

Assessment of stability of double mutant lox against Cre expression

Why did *lox*JTZ17 not increase the frequency of site-specific integration in ES cells? The major difference between the report by Thomson et al. [18] and the present study is the differing species of host cells: prokaryotic cells and eukaryotic cells, respectively. Prokaryotic cells are much smaller than eukaryotic cells. In addition, their genomic DNA is not separated by a nuclear membrane and has a more open structure than eukaryotic cells. Therefore, in prokaryotic cells, Cre proteins and *lox* sites should exist in much higher concentrations and meet and bind more frequently than in eukaryotic cells. The efficiency of integrative recombination in the LE/RE mutant *lox* system depends on the in-affinity of the LE+RE double mutant *lox* site to Cre protein. In this environment of prokaryotic cells, Cre proteins may be able to act on double mutant *lox* sites with incomplete levels of in-affinity for Cre protein. In our assay using ES cells and transient expression of the *cre* gene, the chance of a collision between Cre proteins and the *lox* site was much lower than in prokaryotic cells, and the double mutant *lox* site was exposed to Cre proteins for only a limited time. Therefore, Cre proteins may have disappeared before they could recombine *lox*P and LE+RE double mutant *lox*.

If the ineffectiveness of *lox*JTZ17 recombination in ES cells is due to the limited chance of a collision between Cre protein and *lox* sites, prolonged expression of Cre protein should affect the recognition and recombination of LE+RE double mutant *lox* sites. Therefore, we decided to examine the stability of LE+RE double mutant *lox* sites in ES cells under the continuous presence of Cre protein.

The strategy used to evaluate the stability of the double mutant *lox* is shown in Figure 4. We used a Transthyretine (Ttr) knockout ES line (Ttr-KO41) in which the *lox*71-Pgk-neo-*lox*P-pA-*lox*2272 cassette was inserted into the first exon of the mouse *Ttr* gene. To produce LE+RE double mutant *lox*, six replacement vectors carrying the different RE mutant *lox*-LacZ-pA-Pgk-Pac-loxP were constructed, and the Ttr-KO41 line was coelectroporated with each replacement vector and a Cre-expressing plasmid. We expected that intramolecular recombination would initially occur, resulting in the removal of the Pgk-neo cassette from the genome and separation of the RE-mutant lox-LacZ-Pgk Pac cassette from the plasmid backbone. Cre would then mediate integrative recombination between chromosomal *lox*71 and the RE-mutant *lox* in the cassette. The *Pac* gene in the replacement vector does not have a pA signal; therefore, random integrants should be puromycin-sensitive, and cells should become drug-resistant only upon Cre-mediated targeted integration during which the *Pac* gene fuses to the pA signal. After coelectroporation, puromycin-resistant colonies were selected, and the integration pattern of the LacZ-pA-Pgk-Pac cassette was analyzed by polymerase chain reaction (PCR), Southern blotting, and sequencing (data not shown). Six sub-clones (71/66-P, 71/JTZ-P, 71/KR1-P, 71/KR2-P, 71/KR3-P, and 71/KR4-P) carrying different double mutant *lox* comprising the LE mutation of *lox*71 and the RE mutation of each RE mutant *lox* were established.

We first examined the short-term stability of these double mutant *lox*. Linearized CAG-Cre-IRES-Hyg cassette (pCAGNintCreIH) was introduced into each sub-clone, and Cre-expressing transformants were selected with hygromycin (hyg). Recombination between double mutant *lox* and *lox*P results in removal of the puromycin (puro) resistant genes; therefore, recombined cells become puro-sensitive. If recombination occurs soon after introduction of the CAG-Cre-IRES-Hyg cassette, hyg-resistant (hyg^R) colonies should be puro-sensitive ($puro^S$). In the formation of entire hyg^R $puro^S$ colonies, recombination should occur before the first cell division, or, if formation occurs after the first cell division, recombination should occur in all of the daughter cells. Therefore, in this assay, it is possible to observe recombination within 24-48 h after exposure to Cre protein. To estimate the percentage of such hyg^R $puro^S$ colonies, electroporated cells were divided into two plates. After 5 days of hyg selection, one plate was selected with puro and the other plate was fed with normal medium to obtain the

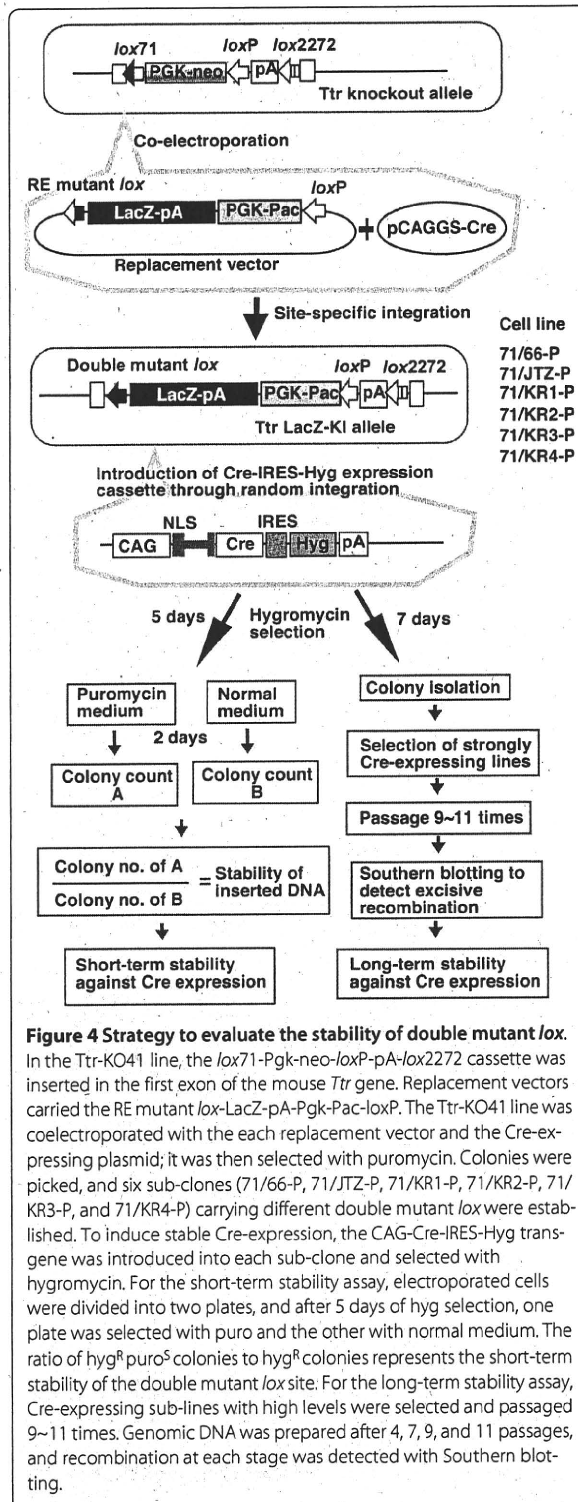

Figure 4 Strategy to evaluate the stability of double mutant *lox*. In the Ttr-KO41 line, the *lox*71-Pgk-neo-*lox*P-pA-*lox*2272 cassette was inserted in the first exon of the mouse *Ttr* gene. Replacement vectors carried the RE mutant *lox*-LacZ-pA-Pgk-Pac-loxP. The Ttr-KO41 line was coelectroporated with the each replacement vector and the Cre-expressing plasmid; it was then selected with puromycin. Colonies were picked, and six sub-clones (71/66-P, 71/JTZ-P, 71/KR1-P, 71/KR2-P, 71/KR3-P, and 71/KR4-P) carrying different double mutant *lox* were established. To induce stable Cre-expression, the CAG-Cre-IRES-Hyg transgene was introduced into each sub-clone and selected with hygromycin. For the short-term stability assay, electroporated cells were divided into two plates, and after 5 days of hyg selection, one plate was selected with puro and the other with normal medium. The ratio of hyg^R $puro^S$ colonies to hyg^R colonies represents the short-term stability of the double mutant *lox* site. For the long-term stability assay, Cre-expressing sub-lines with high levels were selected and passaged 9~11 times. Genomic DNA was prepared after 4, 7, 9, and 11 passages, and recombination at each stage was detected with Southern blotting.

hyg^R colony number. The ratio of hyg^R $puro^S$ colonies to hyg^R colonies represents the short-term stability of double mutant *lox* site.

As shown in Figure 5, all double mutant *lox* showed over 85% stability of inserted DNA, indicating that recombination between double mutant *lox* and *lox*P occurs only slightly over a short time period (24-48 h) in ES cells. Although the/*ox*71/KR3 double mutant showed higher stability than did the others, there was no significant difference among double-mutant *lox* sites (p = 0.12). These results are consistent with the observation that there was no difference in integrative recombination.

In this short-term assay, we could not detect whether recombination occurred later during colony formation in a limited population of the colony because such recombination generates countable, partly puro-resistant ($puro^R$) colonies mixed with recombined $puro^S$ cells and unrecombined $puro^R$ cells. To establish the stability of double mutant *lox* sites against prolonged and strong Cre expression, we cloned and cultured Cre-expressing sub-lines, then examined the level of recombination by Southern blotting. Three double mutant *lox* lines, 71/66-P, 71/JTZ-P, and 71/KR3-P, were selected for the analysis of long-term stability (Figure 4, right side panel). After introduction of the CAG-Cre-IRES-Hyg cassette and colony formation, six Cre-expressing sub-lines from each parental line were isolated and stocked. Cre expression levels in the sub-lines were analyzed by northern blotting (Figure 6a), and the two highest Cre-expressing lines were selected from each double mutant *lox* parental line, as indicated in Figure 6a. The selected lines were then passaged 9~11 times from the original cell stock, and genomic DNA was prepared after 1, 4, 7, 9, and 11 pas-

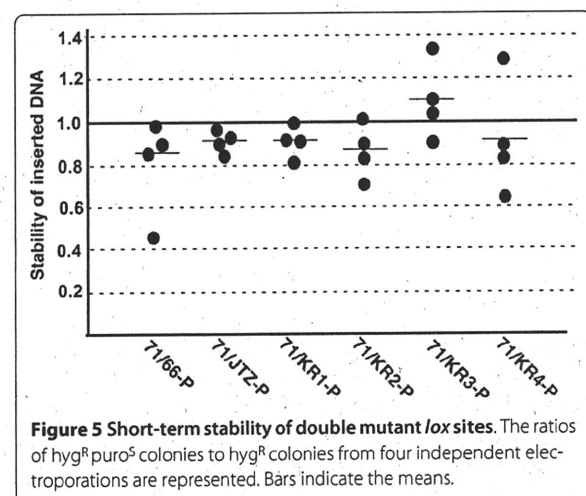

Figure 5 Short-term stability of double mutant *lox* sites. The ratios of hyg^R $puro^S$ colonies to hyg^R colonies from four independent electroporations are represented. Bars indicate the means.

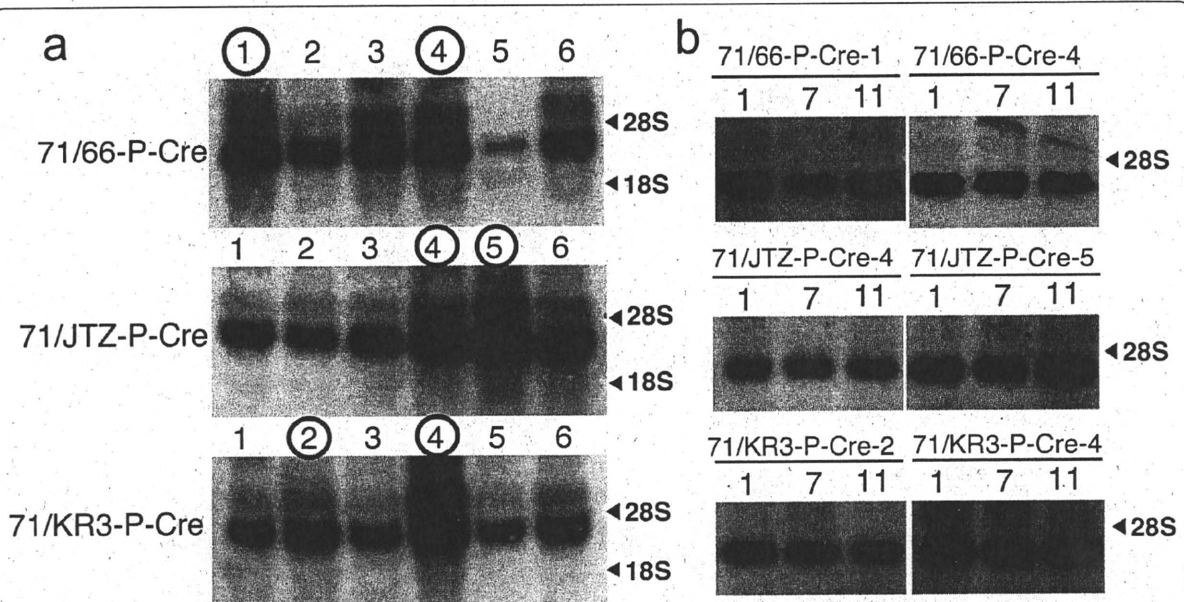

Figure 6 Northern blot analysis of Cre-expressing sub-lines. (a) Six sub-lines carrying the CAG-Cre-IRES-Hyg cassette were isolated from each of three double-mutant *lox* lines: 71/66-P, 71/JTZ-P, and 71/KR3-P. Ten micrograms of total RNA were subjected to electrophoresis and hybridization with a Cre probe. The two highest Cre-expressing lines selected from each of the six sub-lines are indicated by a circle. (b) Selected sub-lines were passaged 11 times, and RNAs from 1st, 7th, and 11th passage were prepared. Four micrograms of total RNAs were subjected to electrophoresis and hybridization with a Cre probe. The expression levels of Cre were maintained during passages.

sages. In order to confirm that the expression level of the *cre* gene was maintained during passage, total RNAs after 1, 7, and 11 passages were also prepared and subjected to northern blotting. As shown in Figure 6b, the *cre* gene was expressed with similar intensity during the 11 passages. Then, recombination between double mutant *lox* and *lox*P sites at each stage was analyzed by Southern blotting, as shown in Figure 7. In 71/66-P-Cre clones, the band for the excised allele was detected clearly at passage one, and the intensity became stronger with increasing passage number (Figure 7a). On the other hand, the bands for the excised allele in 71/JTZ-P-Cre and 71/KR3-P-Cre clones were faint (Figure 7b and 7c), indicating that *lox*71/JTZ and *lox*71/KR3 double mutant *lox* sites were more stable than *lox*71/66.

To estimate the rate of allele excision, band intensities relative to the band derived from the endogenous *Pgk* gene were measured (Figure 7d, solid and hatched lines), and the percentages of excised alleles were calculated (Figure 7d, gray line). In 71/66-P-Cre clones, the rates of allele excision were 48% (clone No. 1) and 74% (clone No. 4), suggesting that the *lox*71/66 double mutant is not highly resistant to re-recombination with *lox*P under continuous exposure to Cre expression. In 71/JTZ-P-Cre and 71/KR3-P-Cre clones, the rates of allele excision were under 21%, meaning that about 80% of double mutant *lox* sites were not recombined, even under the strong Cre expression forced by the CAG promoter (Figure 7d., middle and below).

In order to compare Cre expression levels in these Cre sub-clones, the band intensities shown in Figure 6b were measured by densitometry, and Cre expression level relative to 71/JTZ-P-Cre 4 was calculated. Figure 8 shows a scatter plot of the rate of excision (y-axis) and Cre expression level (x-axis) in each sub-clone. In 71/JTZ-P-Cre and 71/KR3-P-Cre clones, excision rate and Cre expression levels seemed to have a linear relationship with a similar correlation coefficient, suggesting that *lox*71/JTZ17 and *lox*71/KR3 have an in-affinity (stability) level similar to Cre protein. On the other hand, 71/66-P-Cre clones showed 2-4 times higher excision levels than did 71/JTZ-P-Cre and 71/KR3-P-Cre clones.

Thus, *lox*71/JTZ17 and *lox*71/KR3 are highly resistant to the Cre protein, but *lox*71/66 can be recombined with *lox*P when the *cre* gene is expressed strongly and constitutively. Therefore, if we used stable transformants of the *cre* gene for site-directed integration experiments, *lox*66 should show a lower efficiency because of its higher rate of re-excision.

Conclusions

In this study, we screened for RE mutant *lox* sites showing higher recombination efficiency with *lox*71 using ES cells. Although we could not identify any RE mutant *lox* with a

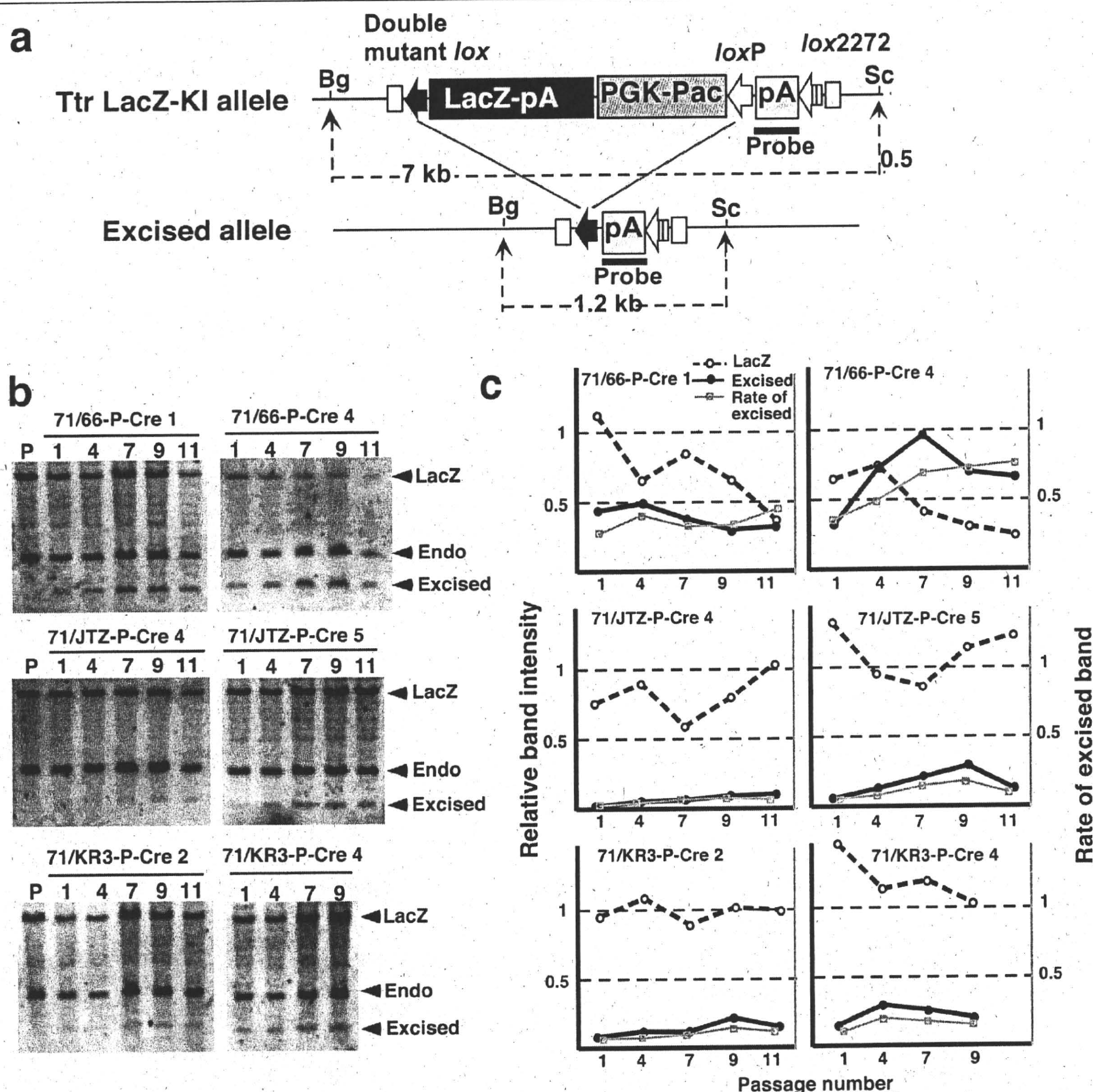

Figure 7 Excising recombination between double mutant *lox* and wild-type *lox*P in strongly Cre-expressing sub-lines. (a) Restriction endonuclease map of the targeted Ttr locus before (Ttr LacZ-KI allele) and after (Excised allele) Cre-mediated recombination. The pA signal of the mouse *Pgk* gene was used as a probe (indicated by the solid line). Fragment sizes are indicated. Bg, BglII; Sc, ScaI. (b) Southern blot analysis of Cre-expressing sub-lines. P represents the parental line before introducing the CAG-Cre-IRES-Hyg cassette. The numbers indicate passage numbers from the original cell stock. The positions of the bands from the nonexcised LacZ allele (LacZ), excised allele (Excised), and endogenous *Pgk* gene (Endo) are indicated by arrowheads. (c) Relative band intensities of the LacZ allele (hatched line) and excised allele (solid line) to the band intensities of the endogenous *Pgk* gene. The rate of excised band [ratio of excised band intensity to total (LacZ + excised) intensity] was calculated and is indicated by the gray line.

significantly higher efficiency than *lox*66, we found that two RE mutant *lox*, *lox*JTZ17 and *lox*KR3, produced more stable (less inactive) double mutant *lox* with *lox*71 than did *lox*66/71. These two mutant RE *lox* sites would therefore be more suitable than *lox*66 for Cre-mediated integration or inversion in ES cells.

Methods

Plasmids

Plasmids pCAGGS-Cre, pCAGlox71bsr, and plox66NZneo have been described previously [17,20]. The *lox*JTZ17 and *lox*KR1, 2, 3 and 4 sequences (Figure 1c) were synthesized, and the *lox*66 sequence of

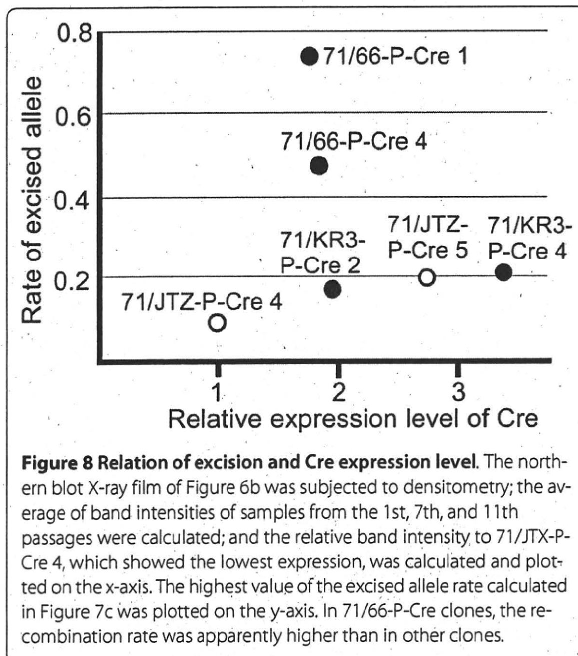

Figure 8 Relation of excision and Cre expression level. The northern blot X-ray film of Figure 6b was subjected to densitometry; the average of band intensities of samples from the 1st, 7th, and 11th passages were calculated; and the relative band intensity to 71/JTX-P-Cre 4, which showed the lowest expression, was calculated and plotted on the x-axis. The highest value of the excised allele rate calculated in Figure 7c was plotted on the y-axis. In 71/66-P-Cre clones, the recombination rate was apparently higher than in other clones.

plox66neo was replaced by these synthesized *lox* sequences to produce pJTZNZneo, pKR1NZneo, pKR2NZneo, pKR3NZneo, and pKR4NZneo (RE *lox*NZneo plasmids). The p66NZPPacP plasmid was constructed by replacing the splice acceptor- enhanced green fluorescent protein (EGFP) cassette of p6SEFPPF [19] into the *LacZ* gene fused with the nuclear localization signal (NLS) derived from the SV40 large T gene (NLS-LacZ). The pJTZNZPPacP, pKR1NZPPacP, pKR2NZPPacP, pKR3NZPPacP, and pKR4NZPacP (RE *lox*NZPacP plasmids) were constructed by replacing the *lox66* sequence with the *lox*JTZ17 and *lox*KR1, 2, 3, and 4 sequences, respectively. The sequences of all *lox* sites in these plasmids were confirmed by DNA sequencing.

The Cre-expression vector, pCAGNintCreIH, was assembled from components of pSP73 (Promega, USA), the CAG promoter [21], the NLS-splice donor (SD)-intron-splice acceptor (SA)-*cre* cassette [22], the internal ribosomal entry site (IRES) from the encephalomyocarditis virus (ECMV), the hygromycin-resistance gene, and the polyadenylation signal (pA) from the mouse *phosphoglycerate kinase-1* (*Pgk*) gene.

ES Cell cultures

ES cells were cultured in KSR-GMEM medium consisting of Glasgow Minimum Essential Medium (GMEM) (Sigma, USA) with 1× MEM nonessential amino acids (Gibco Invitrogen, USA), 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1% fetal bovine serum (FBS; HyClone, Thermo Fisher Scientific Inc., USA), 14% Knockout™ Serum Replacement (KSR; Gibco Invitrogen), and 1100 U/ml leukemia inhibitory factor (LIF; ESGRO, Chemicon, USA). For neutralization of trypsin, FCS-GMEM in which the KSR in KSR-GMEM was replaced with FBS (final concentration, 15% FBS) was used.

ES cell lines (Bs2, Bs17, Bs19, and Bs21) carrying the target lox71 site were established from CGR8 [23] (Gift from Dr. Niwa) by introducing 10 μg of *SpeI*-digested pCAGlox71bsr plasmid DNA. ES cells (3 × 10^6 cells/0.8 ml in PBS) were electroporated using a Bio-Rad Gene Pulser (Bio-Rad, USA) set at 200 V and 960 μF and plated into two 10-cm plates. Blasticidin S selection was started after 48 h of electroporation at 4 μg/ml for 7 days, and colonies were picked, expanded, and stocked. Clones with a single copy integration were selected by Southern blotting analysis.

For Cre-mediated integration, ES cells were coelectroporated with 20 μg of RE *lox*NZneo plasmid and 10 μg of pCAGGS-Cre at 400 V and 250 μF. G418 selection at 600 μg/ml for 7 days was started after 24 h of electroporation. The colonies were then stained with X-gal.

The ES cell line Ttr-KO-41, which carries a *lox*71-Pgk promoter-neomycin phosphotransferase (neo) gene-*lox*P-pA cassette in the first exon of the Ttr gene (*Ttr^{neo}*), has been described previously [24]. To obtain site-specific integrants of RE *lox*NZPacP cassette into the *Ttr^{neo}* allele, Ttr-KO-41 ES cells were coelectroporated with 20 μg of RE *lox*NZPacP plasmid and 10 μg of pCAGGS-Cre at 400 V and 250 μF. Puromycin selection was started after 48 h at 2 μg/ml for 7 days. Colonies were picked and stocked. For electroporation of pCAGNintCreIH, ES cells were electroporated with 20 μg of XhoI-digested pCAG-NintCreIH at 400 V and 250 μF and were fed 150 μg/ml of hygromycin B-containing medium after 24 h of electroporation. Hygromycin B selection was maintained for 5 days; we then changed to puromycin selection at 2 μg/ml or to normal medium for 2 days.

Analyses of DNA and RNA

Cells were lysed with sodium dodecyl sulfate (SDS)/proteinase K, treated with 1:1 (vol/vol) phenol/chloroform, precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 7.5/1 mM ethylenediaminetetraacetic acid (TE). Six micrograms of genomic DNA were digested with appropriate restriction enzymes, electrophoresed in a 0.9% agarose gel, and blotted onto a nylon membrane (Roche, Switzerland). Hybridization was performed using a DIG DNA Labeling Kit (Roche). The intensities of the obtained bands were determined using Printgraph AE-6920-MF (ATTO, Japan).

Total RNA was isolated from ES cells using Sepasol (Nakalai, Japan). Ten micrograms of total RNA were electrophoresed through 1.0% agarose-formaldehyde gels and transferred to a positively charged nylon membrane

(Roche). Hybridization was performed using a DIG RNA Labeling and Detection Kit (Roche).

Statistical analyses

The recombination efficiencies and relative number of blue or white colonies were evaluated by nonrepeated measures analysis of variance (ANOVA). Where a significant difference ($p < 0.05$) was identified, the differences were analyzed further with Student-Newman-Keuls (SNK) tests for multiple comparisons.

Authors' contributions

KA contributed to the project conception and experimental design, carried out the experiments, and drafted the manuscript. YO participated in data production and analysis. MA participated in the experimental design and performed the statistical analysis. KY participated in coordination and helped with writing of the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We wish to thank Ms. Y. Tsuruta for her technical assistance. This work was supported by KAKENHI (Grant-in-Aid for Scientific Research) in Priority Areas "Integrative Research Toward the Conquest of Cancer" (17012018 to K. Y.) from the Ministry of Education, Culture, Sports, Science and Technology.

Author Details

Institute of Resource Development and Analysis, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan

Received: 10 October 2009 Accepted: 31 March 2010
Published: 31 March 2010

References

1. Nagy A: **Cre recombinase: the universal reagent for genome tailoring.** *Genesis* 2000, **26(2)**:99-109.
2. Branda CS, Dymecki SM: **Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice.** *Dev Cell* 2004, **6(1)**:7-28.
3. Voziyanov Y, Pathania S, Jayaram M: **A general model for site-specific recombination by the integrase family recombinases.** *Nucleic Acids Res* 1999, **27(4)**:930-941.
4. Hoess RH, Ziese M, Sternberg N: **P1 site-specific recombination: Nucleotide sequence of the recombining sites.** *Proc Natl Acad Sci USA* 1982, **79(11)**:3398-3402.
5. Fukushige S, Sauer B: **Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells.** *Proc Natl Acad Sci USA* 1992, **89(17)**:7905-7909.
6. Hoess RH, Wierzbicki A, Abremski K: **The role of the loxP spacer region in P1 site-specific recombination.** *Nucleic Acids Res* 1986, **14(5)**:2287-2300.
7. Lee G, Saito I: **Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination.** *Gene* 1998, **216(1)**:55-65.
8. Langer SJ, Ghafoori AP, Byrd M, Leinwand L: **A genetic screen identifies novel non-compatible loxP sites.** *Nucleic Acids Res* 2002, **30(14)**:3067-3077.
9. Bouhassira EE, Westerman K, Leboulch P: **Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange.** *Blood* 1997, **90(9)**:3332-3344.
10. Bethke B, Sauer B: **Segmental genomic replacement by Cre-mediated recombination: Genotoxic stress activation of the p53 promoter in single-copy transformants.** *Nucleic Acids Res* 1997, **25(14)**:2828-2834.
11. Kolb AF: **Selection-marker-free modification of the murine beta-casein gene using a lox2272 [correction of lox2722] site.** *Anal Biochem* 2001, **290(2)**:260-271.
12. Osipovich AB, Singh A, Ruley HE: **Post-entrapment genome engineering: First exon size does not affect the expression of fusion transcripts generated by gene entrapment.** *Genome Res* 2005, **15(3)**:428-435.
13. Albert H, Dale EC, Lee E, Ow DW: **Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome.** *Plant J* 1995, **7(4)**:649-659.
14. Araki K, Araki M, Yamamura K: **Targeted integration of DNA using mutant lox sites in embryonic stem cells.** *Nucleic Acids Res* 1997, **25(4)**:868-872.
15. Zhang Z, Lutz B: **Cre recombinase-mediated inversion using lox66 and lox71: method to introduce conditional point mutations into the CREB-binding protein.** *Nucleic Acids Res* 2002, **30(17)**:e90.
16. Oberdoerffer P, Otipoby KL, Maruyama M, Rajewsky K: **Unidirectional Cre-mediated genetic inversion in mice using the mutant loxP pair lox66/lox71.** *Nucleic Acids Res* 2003, **31(22)**:e140.
17. Araki K, Araki M, Yamamura K: **Site-directed integration of the cre gene mediated by Cre recombinase using a combination of mutant lox sites.** *Nucleic Acids Res* 2002, **30(19)**:e103.
18. Thomson JG, Rucker EB, Piedrahita JA: **Mutational analysis of loxP sites for efficient Cre-mediated insertion into genomic DNA.** *Genesis* 2003, **36(3)**:162-167.
19. Taniwaki T, Haruna K, Nakamura H, Sekimoto T, Oike Y, Imaizumi T, Saito F, Muta M, Soejima Y, Utoh A, *et al.*: **Characterization of an exchangeable gene trap using pU-17 carrying a stop codon-beta geo cassette.** *Dev Growth Differ* 2005, **47(3)**:163-172.
20. Araki K, Imaizumi T, Okuyama K, Oike Y, Yamamura K: **Efficiency of recombination by Cre transient expression in embryonic stem cells: Comparison of various promoters.** *J Biochem (Tokyo)* 1997, **122(5)**:977-982.
21. Niwa H, Yamamura K, Miyazaki J: **Efficient selection for high-expression transfectants with a novel eukaryotic vector.** *Gene* 1991, **108(2)**:193-199.
22. Hashimoto D, Ohmuraya M, Hirota M, Yamamoto A, Suyama K, Ida S, Okumura Y, Takahashi E, Kido H, Araki K, *et al.*: **Involvement of autophagy in trypsinogen activation within the pancreatic acinar cells.** *J Cell Biol* 2008, **181(7)**:1065-1072.
23. Mountford P, Zevnik B, Duwel A, Nichols J, Li M, Dani C, Robertson M, Chambers I, Smith A: **Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression.** *Proc Natl Acad Sci USA* 1994, **91(10)**:4303-4307.
24. Zhao G, Li Z, Araki K, Haruna K, Yamaguchi K, Araki M, Takeya M, Ando Y, Yamamura KI: **Inconsistency between hepatic expression and serum concentration of transthyretin in mice humanized at the transthyretin locus.** *Genes Cells* 2008, **13(12)**:1257-68.

doi: 10.1186/1472-6750-10-29
Cite this article as: Araki *et al.*, Comparative analysis of right element mutant lox sites on recombination efficiency in embryonic stem cells *BMC Biotechnology* 2010, **10**:29

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

International Gene Trap Project: Towards Gene-driven Saturation Mutagenesis in Mice

Masatake Araki[1,*], Kimi Araki[2] and Ken-ichi Yamamura[2]

[1]*Institute of Resource Development and Analysis,* [2]*Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, 860-0811, Japan*

Abstract: While the human genome project has been completed, analysis of functions of each gene is still underway. Knockout and knock down of gene products offer useful tools to understand functions of a single gene *in vivo*. Production of knockout mice using homologous recombination in embryonic stem (ES) cells is a powerful and established strategy. However, it is laborious, time-consuming and expensive if expanding large scale. In mice, the gene trap is an alternative strategy to disrupt gene functions by random disruption of gene. The functions of a gene *in vivo* can be analyzed by production of mice from trapped ES clones. Large-scale gene trap projects have been started in some research centers of the world, and the International Gene Trap Consortium (IGTC) was established to strengthen interactions among centers involved. Moreover, the website of the IGTC has been constructed to integrate information of trap clones from each gene trap project. The database of the IGTC is expanding rapidly because of accumulation of information about gene trap clones from ongoing gene trap projects; approximately 135,000 trapped ES lines are registered in June, 2008. These clones are freely available to academic community. At moment, the IGTC cell lines have covered approximately 10,000 genes in the mouse genome database. Therefore, it is recommended to check the IGTC database before starting knockout experiment, even when annotations of genes are not available. In this review, we introduce principle and short history of gene trap, and then use of the IGTC database is described to obtain trapped ES clones for the experiments.

INTRODUCTION

The house mouse is a useful model organism for biomedical research. Because of small body size and reproductive ability, mice are easy to keep in animal housing environment. In particular, the structural similarity in genome sequences between mice and humans provides interesting findings in functional analysis of the genomes. Furthermore, technology development of genetic engineering in mice offers functional studies of genes in post-genomic era. Here we describe a short history of genetic engineering in mice, mainly focused on gene targeting and gene trap.

In 1989, Capecchi reviewed a new strategy for disrupting a gene of interest (therefore it is called "gene targeting") using homologous recombination in ES cells [1]. Generally, in gene targeting, a part of the coding sequences of the gene are replaced by other DNA sequences containing neomycin resistant gene, which are used for selection of targeted clones in ES cell culture. This exchange leads to disruption of transcripts from the gene targeted as a result. Hence, the gene targeting is a powerful tool to understand functions of genes *in vivo*. Up to now, about ten thousand genes have been knocked out in mice. In 2007, the Nobel Prize in Physiology or Medicine was awarded to Capecchi, Evans, and Smithies for their contribution to gene modifications in mice by using ES cells. The gene targeting strategy is a powerful tool for analyzing *in vivo* functions of genes that have been already cloned. However, the gene targeting cannot be applied for identification of novel genes that have not been cloned, because the homologous recombination needs information of target genome DNA sequences.

The first paper of gene trap was also published in 1989 by Gossler *et al.* who aimed to identify genes which control embryonic development in the mouse [2]. In this paper, they constructed a vector that can monitor expression of an endogenous gene near the integration site. Thereby the vector is called "trap vector". In 1991, Friedrich and Soriano reported an improved promoter trap system [3]. They used both plasmid and retrovirus trap vectors with a novel reporter gene, *β-geo*, encoding a fusion protein with both β-galactosidase and neomycin phosphotransferase activity. Products from the *β-geo* gene enable both drug selection in ES cell culture and visualized tissue localization of trapped genes. Thereafter, Niwa *et al.* described an gene trap method using polyA trap vector [4], and Wurst *et al.* demonstrated a large-scale gene trap screen for developmentally regulated genes in mice [5]. Thereafter, large-scale gene trap projects have expanded worldwide rapidly [3, 5-14]. To organize an efficient network among the gene trap projects, the 1st International Gene Trap Workshop was held in Toronto in 2001. There it was decided to share information of trap clones from each project. For this purpose, novel web-based integrated database was launched since 2004 by the International Gene Trap Consortium (IGTC) consisting of six facilities including us [15]. The database has been regularly updated until now [16-18].

We developed the exchangeable gene trap method [9], and have insisted the importance of post insertional modification from the 1st workshop. Disclosing the database for the

*Address correspondence to this author at the Division of Bioinformatics, Institute of Resource Development and Analysis, Kumamoto University, 2-2-1, Honjo, Kumamoto, 860-0811, Japan; Tel: +81-96-373-6501; Fax: +81-96-373-6502; E-mail: maraki@gpo.kumamoto-u.ac.jp.

1389-2010/09 $55.00+.00 © 2009 Bentham Science Publishers Ltd.

Exchangeable Gene Trap Clones (EGTC) began in August 2004, and it has joined the IGTC database in August 2005. The member of IGTC are using mainly the promoter trap, thus genes expressing in the embryonic stem cells become target.

The International Mouse Knockout Consortium was established to incorporate three mouse knockout projects [19, 20] that are underway worldwide. They agreed on the principles for international collaboration in the area of mouse mutagenesis including (1) free and open release of data and resources generated, (2) the sharing of production plans and information on new technologies and approaches to minimize redundancy and duplication and to promote maximum efficiency, and (3) coordination on archiving and distribution to ensure resources are readily accessible to the scientific community. KOMP (KnockOut Mouse Project) is funded by NIH. This include the acquisition of 251 knockout strains from Deltagen and Lexicon Genetics, support to deposit knockout strains already produced by academic researchers, and creation of 8500 targeted mutations in ES cells using homologous recombination. The project of EUCOMM (European Conditional Mouse Mutagenesis Program) is funded by the EC and the goals of this project are to produce 12,000 conditional gene trap mutations and 8,000 conditional targeted mutations in ES cells. NorCOMM (North American Conditional Mouse Mutagenesis Project) is funded by Genome Canada and the goals of this project are to produce 10,000 gene trap mutations and 2,000 targeted conditional mutations in ES cells. Then, TIGM (Texas Institute for Genomic Medicine) has joined the IKMC (International Knockout Mouse Consortium) as a fourth group [21]. TIGM is developing a mouse ES cell gene trap library in the C57BL/6 genetic background. Currently this library contains over 350,000 clones corresponding to more than 10,000 genes (www.tigm.org).

In this paper, we explain the outline of the gene trap technology, the benefit of the exchangeable gene trap method, and how to use the IGTC data base.

OUTLINE OF GENE TRAP TECHNOLOGY

Mouse ES Cells

The ES cells are indispensable for an extensive functional analysis of the mouse genome. ES cells were originally established from 129/Sv mouse line are widely used for the production of knockout mouse. But also the ES cells are established from various mouse lines such as C57BL/6.

In order to make a knockout vector, it is necessary to use genomic DNA obtained from same mouse strain with the ES cells. The efficiency of homologous recombination is affected by similarity of nucleotide sequence. However, any kind of ES cells can be used in gene trap mutagenesis, because gene disruption is achieved by random integration.

Gene trap is a method to destroy a gene when a trap vector with a drug resistance gene is inserted randomly into the genome of the ES cell. Many gene trap vectors contain a splice acceptor sequence linked to the *lacZ* or *β-geo* reporter gene [22]. The trapped gene can be easily identified using the vector sequence as a tag. For example, 5'-RACE (Rapid Amplification of cDNA Ends), Plasmid rescue, Inverse PCR, and so on. Then an expression pattern of trapped gene can be visualized by staining with chromogenic substrate X-gal, which produces a blue precipitate upon cleavage by β-gal. Moreover, the function at the individual level of the trapped gene can be analyzed by making chimera mouse from the ES cell in which the vector is inserted.

Promoter Trap and polyA Trap

The technique for trapping the gene is roughly divided into two kinds; the promoter trap and the polyadenylation (polyA) trap [3, 4, 11]. In the former method, the drug resistance gene without promoter connected to the downstream of the splice accepter is used in the trap vector. The drug resistance gene will be expressed by the promoter activity when trap vector is inserted in the gene expressed in the ES cells. Trap vector can be inserted into the non-expressing gene of the ES cells. However, these ES cells do not become drug-resistant, thus we can not isolate these ES cells. Thus, drug resistant clones usually reflect gene trapping events, making this approach very efficient for disruption of a gene. In the latter, the drug resistance gene has promoter, but not polyA addition sequence. Therefore, ES cells become drug resistance only when trap vector can utilize polyA addition sequence of the mouse endogenous gene. Advantage of this approach is that non-expressing gene in the ES cells can be trapped. However, disadvantage is that integration sites of polyA trap vector are concentrated into last introns of the trapped genes. Shigeoka *et al.* presented evidence that this remarkable skewing is caused by the degradation of a selectable-marker mRNA used for polyA trapping via an mRNA-surveillance mechanism, nonsense-mediated mRNA decay (NMD) [23]. To overcome this disadvantage, they developed a novel polyA trapping strategy, UPATrap, which suppresses NMD of the selectable-marker mRNA and permits the trapping of transcriptionally silent genes without a bias in the vector-integration site.

Because fusion mRNAs between trapped gene and drug resistance gene are produced, the identification of the trapped gene is easy to do in each method. In the promoter trap, the information of the exon that is located in the upstream of the vector insertion site can be obtained by 5'-RACE. While in the polyA trap, the information of the exon that is located in the downstream of the vector insertion site can be obtained by 3'-RACE.

Plasmid Vector and Retroviral vector

Although plasmid vector can be readily delivered into ES cells by electroporation, multiple copy insertions, concatemers, and deletions of the gene trap vector as well as rearrangements of integration sites are frequently observed. In contrast, retroviral vectors integrate into mouse genome without causing rearrangement of host flanking sequences. Another advantage of retroviral gene traps is that retrovirus-mediated integration tends to occur close to DNase I hypersensitive sites frequently found at the 5' end of genes, which increase the frequency of generating null alleles.

As mentioned above, Friedrich and Soriano showed both of plasmid vector (SAβ-geo) and retrovirus vector (ROSAβ-geo) [3]. Because a polyadenylation signal sequence is included to terminate gene transcription, the gene trap ele-

ments are inserted between the LTRs in reverse orientation relative to viral transcription to avoid interference with virus packaging. ROSA means reverse-orientation-splice-acceptor. Incidentally, ROSAβgeo26 (RASA26) trap mouse line displays ubiquitous expression of the reporter gene during embryonic development and, therefore, has been useful as a marker strain in chimera experiments [24]. Furthermore, Soriano targeted the lacZ gene flanked by loxP sequence at the ROSA26 locus and generated a reporter mouse line for monitoring Cre expression [25].

Although viral vector has several advantages, each vector tends to integrate at specific site of mouse genome. We are using plasmid constructs, since we already established suitable condition for single copy integration by electroporation. Moreover, we devised efficient promoter trap vectors, pU-17, pU-21, pU-21B and pU-21T, which will be describe later.

5'-RACE, Plasmid Rescue and Inverse PCR

In the case of promoter trap, 5'-RACE is generally used for the identification of the trapped gene (Fig. **1A**). RNA is extracted from the embryonic stem cell, the reverse transcriptase reacts with anti sense primer (SP1) in the reporter gene of the trap vector, and 1st strand cDNA is synthesized. After polyA tail addition to the 3'end of 1st strand cDNA, 1st PCR is done with oligo dT-anchor primer and anti sense primer (SP2). 2nd PCR is continuously done with anchor primer and SP3 primer. The sequence of 2nd PCR product (RACE product) is determined by SP4 primer. It should be a fusion transcript of trapped gene and reporter gene.

However, it is necessary to determine the genomic sequence which is adjacent to the inserted trap vector in order to distinguish the homozygote from the heterozygote.

One of the methods to clone the genomic fragment that is adjacent to the vector is a plasmid rescue (Fig. **1B**). This method utilizes the plasmid DNA with replication origin in the trap vector. It contains three steps; (i) digest with a suitable restriction enzyme (RE), (ii) self-ligation, and (iii) transformation of the E. coli.

On the other hand, inverse PCR is a method to isolate the fragment that is adjacent to the vector (Fig. **1C**). First two steps of inverse PCR are common with plasmid rescue. That includes (i) digestion with a suitable restriction enzyme (RE), and (ii) self-ligation. Then, PCR is done with out-sided primers of trap vector.

Application of Mouse Genome Browser for the Annotation of Trapped Gene

We are mainly using BLAT (BLAST-like Alignment Tool) search of UCSC Genome Browser to determine the trapped gene. BLAT on DNA is designed to quickly find sequences of 95% and greater similarity of length 25 bases or more. Fig. (**2**) shows an example of BLAT search result for the 5'-RACE product of the Ayu21-T154 gene trap clone. You can choose "Mouse" in "Genome" and the newest version in "Assembly", and input "Query" sequence (5'-RACE product), then "submit". Because "Browser" of the width of the Query sequence is displayed first when the "browser" button of "ACTIONS" is pushed, it is better to adjust by suitably "zoom out", or changing the figures in the "position/search" box. Moreover, Browser is customized by changing the display method of various Track. Especially, when "Gene Trap" in "Genes and Gene Prediction Tracks" is made "Pack", the trap clone registered in IGTC can be displayed. In Fig. (**2**), it is understood that the trap vector has been inserted in the downstream intron of the second exon (ATG exon) of the ankyrin repeat domain 47 (Ankrd47) gene.

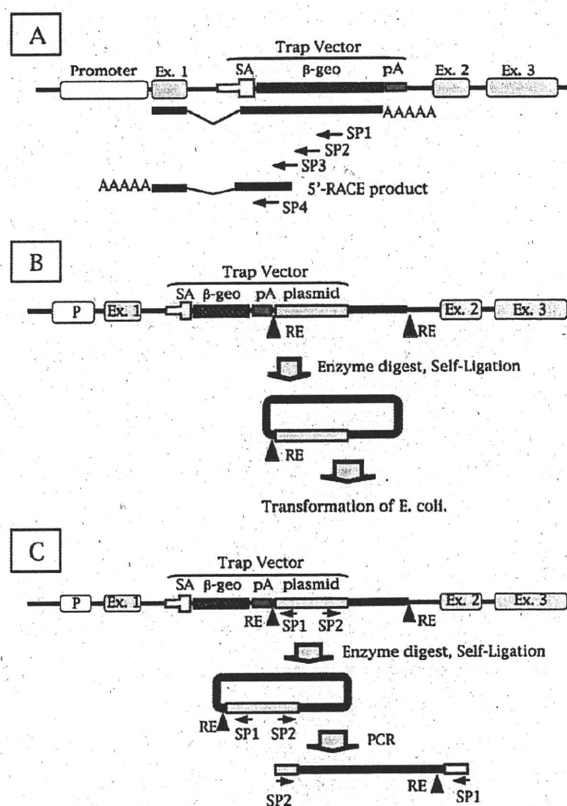

Fig. (1). Techniques for the identification of the trapped gene. (A) 5'-RACE (Rapid Amplification of cDNA Ends). Ex.; Exon, SA; splice acceptor, SP1~SP4; gene specific anti-sense primer. (B) Plasmid rescue. RE; restriction enzyme. (C) Inverse PCR.

The Ensemble Contig View is also a useful genome browser. Ensemble is a joint project between EMBL – European Bioinformatics Institute (EBI) and the Wellcome Trust Sanger Institute (WTSI) to develop a software system which produces and maintains automatic annotation on selected eukaryotic genomes. Furthermore Mouse Genome Informatics (MGI) of the Jackson Laboratory should be checked for mouse research. MGI is the international database resource for the laboratory mouse, providing integrated genetic, genomic, and biological data to facilitate the study of human hearth and disease.

Website Information

UCSC Genome Bioinformatics

[http://genome.ucsc.edu/index]

Fig. (2). BLAT search of UCSC Genome Browser.
BLAT on DNA is designed to quickly find sequences of 95% and greater similarity of length 25bases or more. BLAT; The BLAST-like Alignment Tool.

Ensemble [http://www.ensembl.org/]

Mouse Genome Informatics

[http://www.informatics.jax.org/]

EGTC [http://egtc.jp]

IGTC [http://www.genetrap.org]

CARD R-BASE

[http://cardb.cc.kumamoto-u.ac.jp/transgenic/]

DATABESE FOR THE EXCHANGEABLE GENE TRAP CLONES (EGTC)

Cre - Mutant Lox System

A precise mutation such as point mutations and gain-of-function mutation can not be introduced by conventional gene targeting or gene trap method. We solved this problem by applying the Cre-lox system and developed "the exchangeable gene trap method"[9]. Cre-lox system obtained from Bacteriophage P1 [26-28]. Cre is an enzyme recognizing a sequence motif of 34 bp, called loxP. The loxP sequence is composed of an asymmetric 8 bp spacer flanked by 13 bp inverted repeats. Cre protein bind to the 13 bp inverted repeat mediating the recombination within the 8 bp spacer. If a DNA fragment is flanked by two loxP sites in the same orientation, Cre excises this DNA fragment, leaving a single loxP site behind. Since null mutation often causes embryonic lethality, Rajewsky *et al.* devised conditional gene targeting strategy using Cre-loxP system [29, 30].

We reported that this Cre-loxP system worked efficiently in mouse fertilized eggs [31]. Then we developed the knock-in system in the mouse ES cell using mutant lox [32]. Mutant loxs can be roughly divided into two category, "LE/RE mutant lox" and "Heterospecific lox". In the left element (LE) mutant, lox71, five bases are substituted in the left side, and in the right element (RE) mutant, lox66, five bases substituted in the right side [33]. Recombination between a chromosomally located lox71 site and a lox66 site on a targeting plasmid results in site-specific integration of the plasmid producing a double (LE + RE) mutant lox site and a wild-type loxP site. Since the binding affinity of the LE + RE lox mutant site for Cre recombinase is reduced, the integrated plasmid is stably retained. On the other hand, heterospecific lox sites have mutation(s) in the 8 bp spacer region [34]. Several groups have used lox511, which contains a single base substitution, and demonstrated successful gene replacement [35-37]. Lee and Saito developed two other heterospecific mutant lox sites with two base substitution, lox2272 and lox5171[38]. They showed that the mutant sites never recombined with the wild-type loxP site, while lox511 can recombine with loxP at low frequency using an *in vitro* system. We demonstrated site-directed integration of the Cre gene mediated by Cre recombinase using a combination of "LE/RE mutant lox" and "Heterospecific lox"[39].

Fig. (3) shows the outline of "The Exchangeable Gene Trap System" using these Cre-mutant lox system. Trap vector is introduced into ES cells by electroporation. Then G418-resistant colonies are isolated. Trapped genes are analyzed by 5'-RACE. Trapped clone, in which gene X is disrupted, is subjected to chimera production, followed by es-

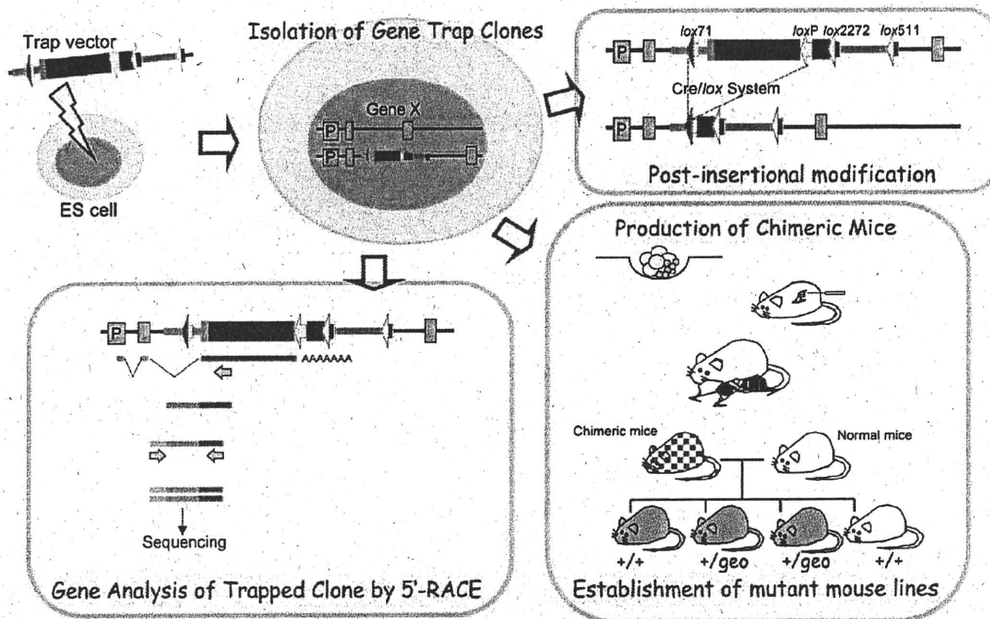

Fig. (3). The exchangeable gene trap system.
Trap vector including the drug resistance gene is introduced into ES cells by electroporation. Drug resistant colonies are picked up and expanded. Trapped gene (gene X) is analyzed by 5'-RACE. The trapped clone is subjected to chimera production. Gene X disrupted mouse line is established and analyzed for its phenotype. Finally we can carry out post-insertional modification. For example, the phenotype of Gene X disruption may be rescued by deletion of splice acceptor and drug resistant gene of trap vector by Cre/lox system.

tablishment of mutant mouse line and analysis of mutant phenotypes. One characteristic feature of this method is that we can carry out post-insertional modification, for example knock-in of Y gene cDNA to express under control of X gene promoter.

Promoter Trap

We devised efficient promoter trap vectors, pU-17, pU-21, pU-21B and pU-21T, to produce a null allele. We inserted the three stop codons in splice acceptor, which are located upstream of the ATG of the reporter gene, *β-geo*, and did not use the IRES (internal ribosome entry site) sequence. If this vector was inserted into the downstream of endogenous gene, translation would stop at the stop codon. As the gene does not have IRES sequence, the reporter gene would not be translated independently. Thus, ES clones will not become drug-resistant and such clones will not be isolated.

Post Insertional Modification

Fig. (4) shows the example of post insertional modification using our gene trap clone. First, we induce recombination between lox71 and loxP by Cre recombinase resulting in deletion of the splice acceptor and the reporter gene (β-geo). By this treatment we expect recovery of transcription from the endogenous gene, thus enabling it to confirm the relationship between the gene disruption and phenotype observed in knockout mice. Second, a gene of interest can be inserted between lox71 and loxP using a plasmid containing a gene of interest or other reporter gene flanked by lox66 and loxP [39]. Even the Cre gene can be inserted to produce Cre-driver mouse [40].

Establishment of Mouse Lines and CARD R-BASE

We are making chimera mice using the aggregation method from the trap ES cells registered in EGTC. ES cells are aggregated with morulae from ICR mice. We usually use 125 morulae per one ES clone. Chimeric mice are mated with C57BL/6 females. Genomic DNAs of F1 progenies and original ES cells are subjected to Southern blotting to confirm whether the integration pattern of the vector is identical between the mouse line and the original ES clone. The established mouse line is deposited to CARD R-BASE; the database for cryopreserved embryos. Center for Animal Resources and Development (CARD) is one of founding members of FIMRe (Federation of International Mouse Resources) like Jackson Laboratory, EMMA (European Mutant Mouse Archives) and MMRRC (Mutant Mouse Regional Resource Centers) [41], and CARD can supply living mice or frozen embryos established from the ES clones registered in EGTC. By June of 2008, total 469 ES cell lines have been registered into the EGTC. Among them, 170 mouse lines have been established and deposited to CARD.

INTERNATIONAL GENE TRAP CONSORTIUM (IGTC)

Members of IGTC

The 9 members of IGTC as of April, 2008 are as follows; BayGenomics (USA), Center for Modeling Human Disease (CMHD, Toronto, Canada), Embryonic Stem Cell Database (ESDB, University of Manitoba, Canada), Exchangeable Gene Trap Clones (EGTC, Kumamoto University, Japan), German Gene Trap Consortium (GGTC, Germany), Sanger

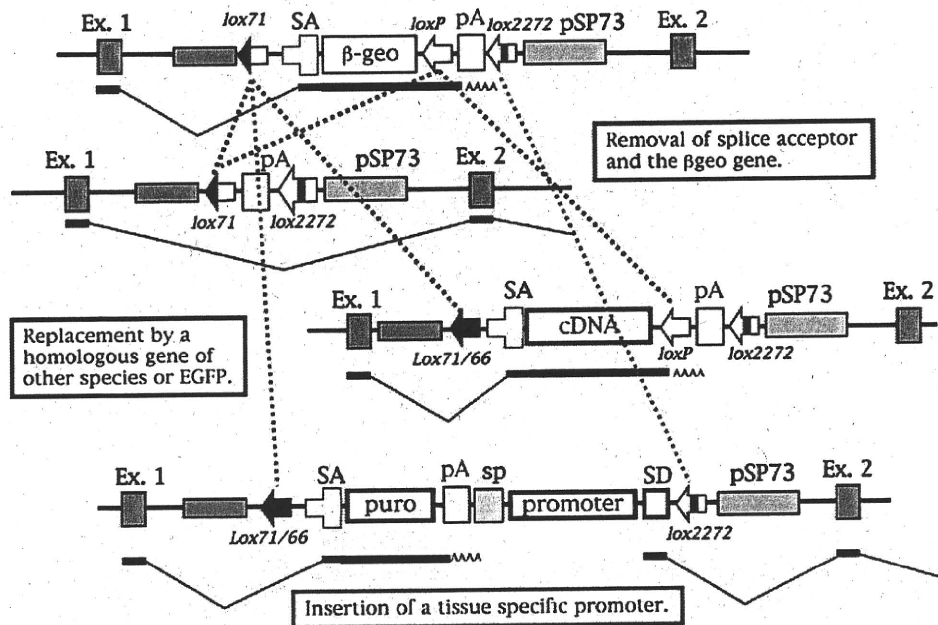

Fig. (4). Post insertional modification.
(1) Removal of splice acceptor and *β-geo* gene. (2) Replacement by a homologous gene of other species or EGFP. (3) Insertion of a tissue specific promoter. Ex. 1 and Ex. 2; Exon 1 and Exon 2 of the trapped gene, pA; polyadenylation signal, SA; splice acceptor, sp; spacer, promoter; tissue specific promoter, SD; splice donor.

Institute Gene Trap Resource (SIGTRA, Cambridge, UK), Soriano Lab Gene Trap Database (Fred Hutchinson Cancer Research Center, Seattle, USA), Texas Institute for Genomic Medicine-TIGM (TIGM, USA), and TIGEM-IRBM Gene Trap (TIGEM, Naples, Italy).

IGTC Database

About 133,000 ES trap clones are registered by April, 2008. This covers 8970 (32.17%) genes in the annotation of Ensemble and 11,344 (17.89%) genes in the annotation of Entrez.

IGTC website is composed of "Information", "Data Access", "Tutorials" and "Request ES Cell Lines". In the "Information", overview of IGTC project, experimental protocols, software, workshop, IGTC members, publications, FAQ, news archive, and links are described. Various search tools are prepared for Data Access. "Tutorials" gives a brief overview of gene trap technology, reviews gene trap vector types and function, and discusses experimental opportunities available to gene trap cell line users.

Keyword/ID Search

When you want to find the trap clone in which a gene of interest is disrupted, we recommend Keyword/ID Search in "Data Access". One example is shown in Fig. (5) where "Ctbp (C-terminal binding protein)" is used as a key word. The display of "In Situ" means you can obtain "In Situ Image" which started in February, 2008. If you click C-terminal binding protein1 in Gene Description, the page of Gene Annotation will open. Please press triangle button of "Identified Cell Lines", you can find five Ctbp1 trap clones with the Identification Status, Source, and Vector. In the Gene Annotation page, you can also find the other information such as Synonyms, Entrez Gene, Ensemble Gene Report, Accessions (RefSeq, MGI, Protein, EMBL, UniGene, and *et al.*), Pubmed, Homology, Gene Ontology (GO), InterPro, Phenotypes, and OMIM. Moreover, Sequence Alignment Image shows the integration site of trap vector in Ctbp1 gene.

Gene Ontology

If you choose "Gene Ontology" in Search Field of "Keyword/ID Search" in "Data Access", you will hit 32 genes when searching the term "anti-apoptosis". The Gene Ontology aims to provide a controlled vocabulary that can be used in any organism. GO Term is divided into three parts (biological process, cellular component, and molecular function) and anti-apoptosis (GO:0006916) is included in biological process. 42 GO terms are described on the Gene Annotation page of Bcl2 and it is linked to the page to which the GO database (AmiGO) corresponds respectively. It is one of the important tools to quickly acquire information on the function of the trapped gene.

Biological Pathways

It is also possible to find a trap clone related to your topics of research from "Biological Pathways" which is listed on the bottom of "Data Access". For example, choose "Mm_Apoptosis.mapp" listed on the top. The number of each gene shown in the map of Biological Pathway is assigned according to the number of trap clones registered in IGTC. For example, while the number of Bcl2 trap clone is only 2, 57 Bcl2l2 trap clones are registered in the IGTC.