Asian-Aust. J. Anim. Sci.
Vol. 21, No. 5 : 745 - 753
May 2008

www.ajas.info

Recent Progress in Biotechnology-based Gene Manipulating Systems to Produce Knock-In/Out Mouse Models

Woon Kyu Lee*, Joong Jean Park[1], Seok Ho Cha[2] and Cheol-heui Yun[3]

Laboratory of Animal Medicine, Medical Research Center, Yonsei University Health System, Seoul 120-752, Korea

ABSTRACT : Gene-manipulated mice were discovered for the first time about a quarter century ago. Since then, numerous sophisticated technologies have been developed and applied to answer key questions about the fundamental roles of the genes of interest. Functional genomics can be characterized into gain-of-function and loss-of-function, which are called transgenic and knock-out studies, respectively. To make transgenic mice, the most widely used technique is the microinjection of transgene-containing vectors into the embryonic pronucleus. However, there are critical drawbacks: namely position effects, integration of unknown copies of a foreign gene, and instability of the foreign DNA within the host genome. To overcome these problems, the ROSA26 locus was used for the knock-in site of a transgene. Usage of this locus is discussed for the gain of function study as well as for several brilliant approaches such as conditional/inducible transgenic system, reproducible/inducible knockdown system, specific cell ablation by Cre-mediated expression of DTA, Cre-ER^{TM} mice as a useful tool for temporal gene regulation, MORE mice as a germ line delete and site specific recombinase system. Techniques to make null mutant mice include complicated steps: vector design and construction, colony selection of embryonic stem (ES) cells, production of chimera mice, confirmation of germ line transmission, and so forth. It is tedious and labor intensive work and difficult to approach. Thus, it is not readily accessible by most researchers. In order to overcome such limitations, technical breakthroughs such as reporter knock-in and gene knock-out system, production of homozygous mutant ES cells from a single targeting vector, and production of mutant mice from tetraploid embryos are developed. With these upcoming progresses, it is important to consider how we could develop these systems further and expand to other animal models such as pigs and monkeys that have more physiological similarities to humans. (**Key Words :** Transgenic, Knock In/Out, ROSA26, Tetraploid, ES Cells, Gene Manipulation)

INTRODUCTION

More than a quarter of a century has passed since gene-manipulating systems were developed in mice (Evans and Kaufman, 1981; Hudziak et al., 1982; Koller and Smithies, 1989; Capecchi, 2005). Until now, numerous technical approaches were reported to upgrade the systems. At the beginning, most cases belong to just a trial with basic strategies to see the phenotypic changes only in mice. However, with this approach, we have very limited understanding on the functions of the targeted genes which are still unresolved. As the importance of gene functions in the context of the host genome is being recognized rapidly, there are limitations in explaining true values of certain genes from *in vitro* study to make a link to *in vivo* functions. Here, we give a brief overview on the gene-manipulating systems available and introduce new strategies developed recently together with some of on going trials in our laboratories.

OVERVIEW OF GENE-MANIPULATING SYSTEMS IN MICE

Functional studies of a certain gene involve adding the gene (gain of function) to or deleting the gene (loss of function) from their host genome. The former is called transgenic and the latter is called knock out, respectively. Once the transgenic vector is constructed, it is injected into the pronucleus, usually male's because of its larger size, using a micromanipulator. Then, the DNA-injected embryos are transferred into the oviduct (embryos at 1-cell to morula

* Corresponding Author: Woon Kyu Lee. Tel: +82-2-2228-2721, Fax: +82-2-362-8647, E-mail: wklee@yuhs.ac

[1] Department of Physiology, College of Medicine, Korea University, Seoul 136-705, Korea.

[2] Department of Pharmacology and Toxicology, and Center for Advanced Medical Education by BK21 Project, College of Medicine, Inha University, Incheon, Korea.

[3] Graduate School of Agriculture Biotechnology, College of Agriculture and Life Science, Seoul National University, Seoul 151-921, Korea.

Received September 1, 2007; Accepted December 13, 2007

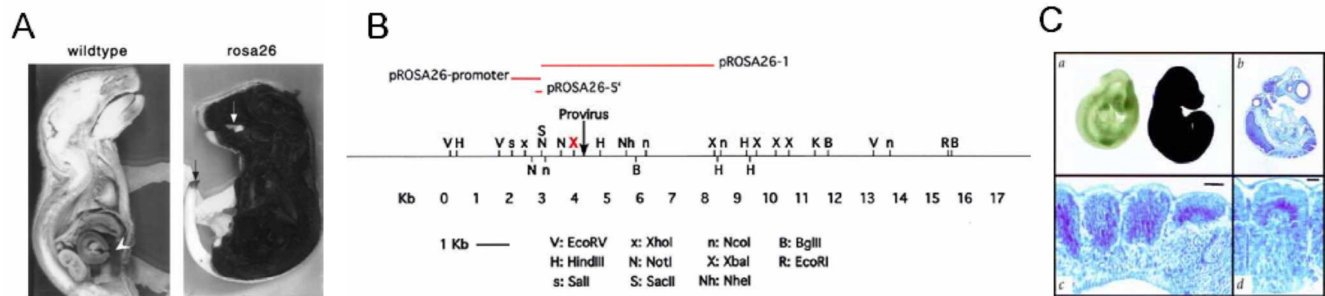

Figure 1. ROSA26 locus. (A) Ubiquitous expression of β-gal in ROSA26 newborn mice. (B) Map of ROSA26 locus. Arrow indicates the site of provirus infection. Red X is XbaI for knock-in site. (C) Generalized LacZ expression following Cre recombination. (a) Whole-mount X-Gal staining of E9 R26R heterozygous (*left*) and R26R/R26Cre (*right*) compound heterozygous embryos. (b) Sagittal section. (c) Cross-section through a somite (×200). (d) higher magnification of c (×1,000). Reprinted with permission of (a) Zambrowicz et al. (1997), (b) http://www.fhcrc.org/science/labs/soriano/home.html, (c) Soriano (1999).

stage) or uterus (embryos at blastocyst stage) of pseudo-pregnant female mice. The pups need to be genotyped to confirm if the foreign DNA is inserted by using the polymerase chain reaction (PCR) or Southern blot. The positive ones are back-crossed to get germ line transmitted mice called founders which contain a hemizygous insertion. The basic components of the vector for the transgenic systems consist of an ORF (open reading frame) of a gene, a promoter (minimal promoter or positive/negative regulator), and the RNA processing components (splicing acceptor and poly (A) signal). There are many different kinds of promoter elements for the selection as well as RNA processing components. Precise control of these components is becoming critical issues because this will provide the way to make tailor-made transgenic mice to answer the given questions properly.

On the other hand, precise design and construction of a targeting vector to knock-out a gene are also necessary. The basic strategy in this system is to delete out the arm that is important for the gene function by the homologous recombination using the 5' and 3' fragment on each side of it. It is usual to use two different markers for the selection of a positive embryonic stem cell (ES) colony. The technique is called positive and negative dual selection. After getting the positive ES cells, it is introduced into the blastocysts and transferred to the pseudo-pregnant mice. The ES cells from the mice which have dominant hair color to the recipient mice are usually used. It is easy to confirm whether the introduced ES cells contributed to the host body color as a complexity of chimera. Like the transgenic, it needs to be back-crossed with wild-type mice to verify the germ line transmission, and the pups that are positive in genotyping will be heterozygous mutants.

SEVERAL POINTS OF CONSIDERATION

Gain-of-function studies

Currently, the most popular way to generate transgenic mice is to inject a constructed vector into the pronucleus of an embryo using a micromanipulator. This is a very simple and highly efficient method especially when we consider the time required, cost and labor. But there are critical drawbacks in this system when one raises in-depth questions on the gene function. First of all, the injected vector is integrated most likely randomly to the host genome. It is virtually indistinguishable whether the phenotype is caused by addition of a gene, or by disturbance of the host gene at the region where the foreign gene is introduced (called position effect). So, it needs at least 3 different transgenic lines to confirm true transgenic results. Second, the foreign gene can be integrated into the host genome with variable copy numbers (anywhere from one to numerous copies). Therefore, the acceptable expression levels of a transgene for its function and phenotype are uncertain. Third, by using this method, the transgene is instable in the transgenic mice. We do not know exactly why it happened since it is impossible to explain the mechanisms on how the transgene is integrated into the host genome. Based on these potential problems, the transgenic mice obtained by using the microinjection technique can give us only partial information of a gene together with a certain level of uncertainty.

Knock-in system using the ROSA26 locus

To overcome the downside of the microinjection technique, a new knock-in method was developed for the gain-of-function study using the ROSA26 locus (Figure 1B). The provirus has been shown to interrupt two transcripts that encode a nuclear RNA and its expression is ubiquitous (Figure 1A) (Zambrowicz et al., 1997). With the characterization of this locus, it has been a useful tool to make transgenic mice expressing a transgene in a generalized fashion. It is possible to use the genomic locus for target genes as a knock-in site (Soriano, 1999). Since it was approved, it has been used in many laboratories to produce a variety of knock-in lines (Murtaugh et al., 2003;

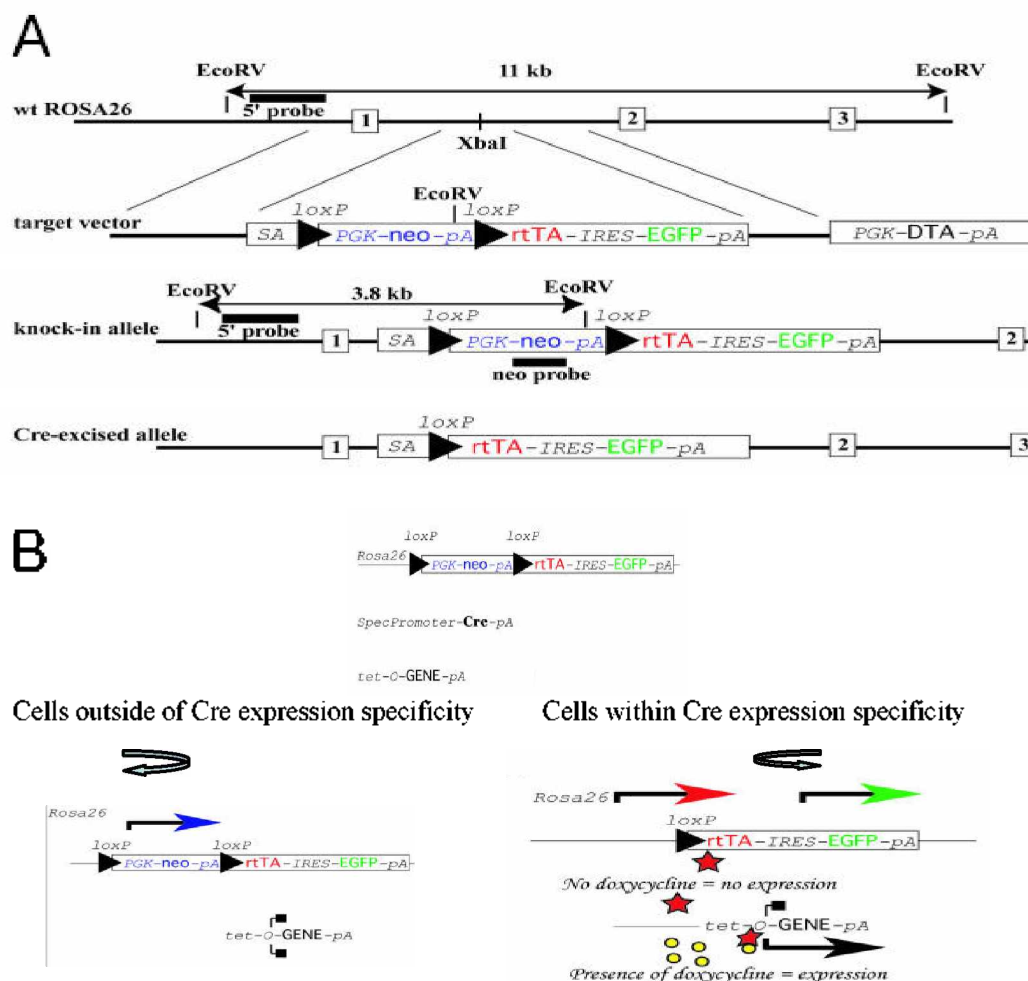

Figure 2. The strategy of conditional and inducible transgenic system. (A) Targeting strategy. The exons of the ROSA26 gene are listed as numbered boxes. EGFP is used as a reporter for Cre recombination. 5' probe for Southern blot and loxP site (triangles) are also shown. (B) Operation of the Cre/loxP-dependent, tetracycline inducible transgenic system. Reprinted with permission of Belteki et al. (2005).

Gu et al., 2005; Jeong et al., 2006) including a Cre reporter line expressing conditional lacZ (*Gtrosa26*^{tm1Sor}; Figure 1C; http://www.fhcrc.org/science/labs/soriano/home.html) (Soriano, 1999). It is to be noted that some of the lines developed are available from the Jackson Laboratory, USA.

Targeting to the ROSA26 locus is conveniently achieved by introducing a desired gene into the first intron of the locus, at its unique XbaI site, approximately 248 bp upstream of the original gene trap line (Figure 1B). The pBigT vector was developed for efficient cloning which has the PacI/AscI site. Also, the XbaI site of pROSA26-1 is replaced with PacI/SwaI/AscI site for the cloning of the pBigT vector (Srinivas et al., 2001). A neomycin resistance cassette for positive selection and a diphtheria toxin gene for negative selection are also included in the targeting vector that allows high rates of homologous recombination (usually ~25-50% of G418^r clones). There is also a flanking probe (pROSA26-5' or the 337 bp Not I fragment of the pROSA26 promoter) for genotyping as well as oligos for PCR selection (http://www.fhcrc.org/science/labs/soriano/home.html). One of the useful applications with the ROSA26 locus is the promoter for broad expression in mice (Kisseberth et al., 1999). It has been developed in many laboratories and showed an ubiquitous activity even though the expression was not uniform (Grippo et al., 2002; Odorfer et al., 2007). After the ROSA26 locus was developed as a knock-in site for gain of function, many types of mouse lines were developed and the followings are some examples as representatives on various settings.

Conditional/inducible transgenic system : A triple transgenic mouse system combines the tissue specificity of any Cre-transgenic lines with the inducibility of the reverse tetracycline transactivator (rtTA)/tetracycline-responsive element (tet-O)-driven transgenes (Belteki et al., 2005; Yu et al., 2005). To ensure reliable rtTA expression in a broad range of cell types, the rtTA transgene was knock-in into the ROSA26 locus (Figure 2A). As shown in Figure 2B, mice that are homozygous for the targeted insertion of the

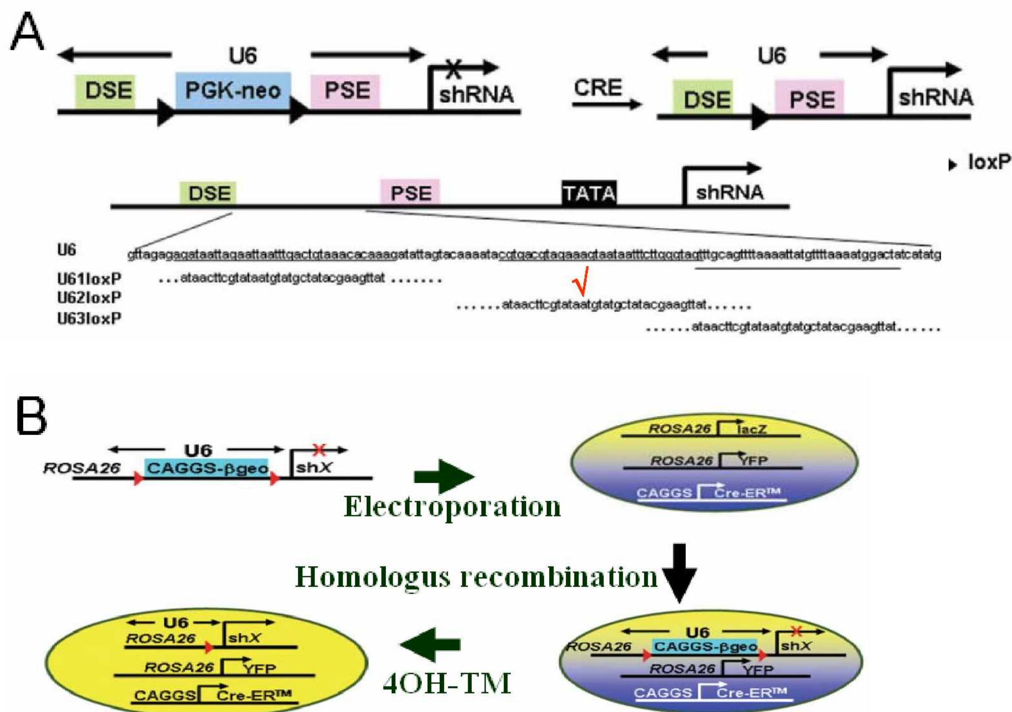

Figure 3. The strategy for the inducible shRNA expression system. (A) Insertion of loxP sequence in the U6 promoter does not affect U6 promoter activity. A loxP sites-flanked PGK-neo fragment replaces a 34-bp sequence between the distal and proximal sequence elements (DSE and PSE, respectively) with in the U6 promoter, blocking shRNA transcription. (B) Introduction and activation of inducible shRNA expression at the ROSA26 locus in YFP3.1 cells. Reprinted with permission of Yu and McMahon (2005).

conditional rtTA-IRES-EGFP bicistronic transgene at ROSA26 locus can be bred with double transgenic mice that carry a tissue-specific Cre transgene and a doxycycline-inducible rtTA-dependent *tet-O*-GENE. A total of 25% of the pups will be triple transgenic (SpecPromoterCre$^{Tg/+}$, ROSA26-STOP-rtTA-IRES-EGFP$^{Tg/+}$, *tet-O*-GENE$^{Tg/+}$). In cells that do not express Cre, neither rtTA nor EGFP protein can be generated, which means that the *tet-O*-GENE is silent. In Cre-expressing cells, on the other hand, rtTA and EGFP expression is turned on. However, it is important to note that in the absence of an inducer (e.g., doxycycline), rtTA cannot activate the expression of the *tet-O*-GENE whereas the addition of doxycycline results in the formation of an active transactivator and the expression of the target gene.

Reproducible/inducible knockdown system : By using the inducible shRNA cassette in the ROSA26 locus of the mouse, it was possible to generate reproducible and controlled expression of shRNA to produce discernable phenotypes *in vitro* and *in vivo* (Yu and McMahon, 2006; Hitz et al., 2007) (Figure 3A). As shown in Figure 3B, an inducible shRNA expression cassette was placed into the ROSA26 knock-in site. The "floxed" CAGGS-β-geo allows the identification of the targeted ES cells by neo resistance as well as by the loss of β-gal activity through Cre-mediated recombination. The vector was introduced into YFP3.1 cells (Mao et al., 2005) to replace the original lacZ allele of the ROSA26 locus, in which β-gal activity in targeted ES cells is under the control of Cre recombinase. In the presence of 4OH-tamoxifen (4OH TM), Cre-mediated recombination occurs, which activates shRNA expression. It can bypass the prescreening of random integration in ES cell clones and further enable conditional gene knockdown with temporal and/or tissue specificity.

Specific cell ablation by Cre-mediated expression of DTA : A versatile system to specifically ablate cells of any lineage during embryogenesis or in adulthood can give us substantial benefits for studying development and model for human diseases of various etiologies. A ROSA26-eGFP-DTA mouse line, which combines the use of enhanced green fluorescent protein (EGFP), diphtheria toxin A subunit (DTA), and Cre recombinase is generated for this purpose (Ivanova et al., 2005). Mice carrying this construct are normal and fertile, indicating the absence of DTA expression. However, upon Cre-mediated excision of the floxed region, DTA expression is activated resulting in the specific ablation of Cre-expression cells. As an example of this approach, Nkx2.5 and Wnt1-expressing cells are ablated by using the Nkx2.5-Cre and Wnt1-Cre mouse lines, respectively (Ivanova et al., 2005). This approach will

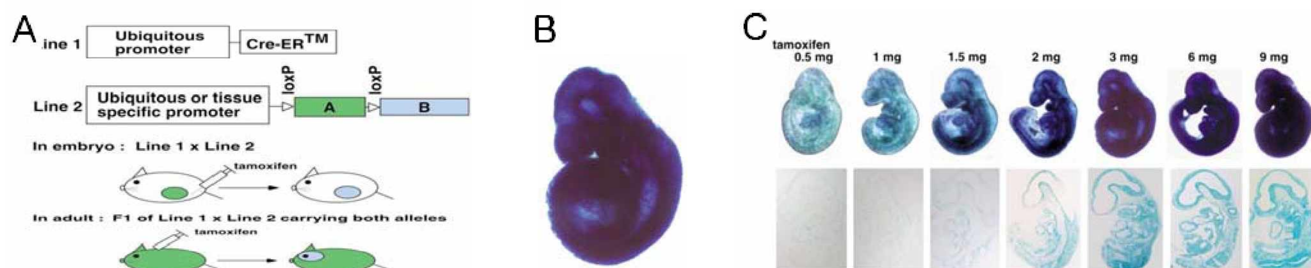

Figure 4. Usage of a TM-inducible gene modification system. (A) Potential use of CAGGCre-ER^{TM} line to regulate gene activation or inactivation in an embryo or postnatal animal. (B) Whole-mount views of 9.5-dpc embryos following histochemical staining of embryos for β-galactosidase activity following administration of TM at 8.5-dpc. (C) Dose-dependent TM inducing recombination in CAGGCre-ER^{TM};R26R mouse embryos. Compound transgenic embryos were subjected to whole-mount and 6-μm section histochemical staining for β-galactosidase activity at 9.5-dpc, 24 h after administration of the indicated dose of TM administration. Reprinted with permission of Hayashi and McMahon (2002).

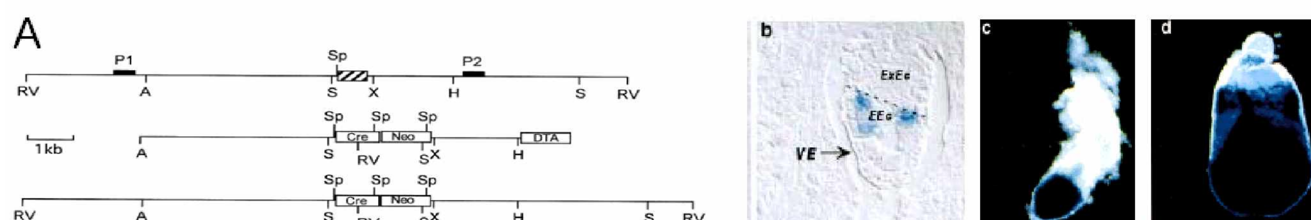

Figure 5. Insertion strategy of Cre into the Mox2 gene as well as its expression in R26R mice. (A) Map of Cre knock-in scheme. The hatched box represents the first coding exon. NLS-Cre-bpA (bovine growth hormone polyadenylation sequence) was inserted in frame in the targeting vector by PCR mutagenesis at the SphI site present in the Mox2 genomic locus. (B) Cre expression pattern in R26R mice. Cre expression first detected at E5.5 in epiblast cells, sagittal section. The dashed line represents the presumptive separation of extra-embryonic and embryonic ectoderm. C/D) Whole mount E6.5 and E7.5 embryos, respectively. Reprinted with permission of Tallquist and Soriano (2000).

provide a very useful resource for the genetic ablation of specific groups of cells.

Cre-ER^{TM} mice: a useful tool for temporal gene regulation : Conditional gene inactivation using the Cre/loxP system is widely used currently, but the difficulty in proper regulation of Cre expression remains one of the bottlenecks in this field. One approach to regulate Cre activity utilizes a mutant estrogen hormone binding domain (ER^{TM}) to keep Cre inactive unless the non-steroidal estrogen analog 4-hydroxytamoxifen (4OH-TM) is present (Hayashi and McMahon, 2002) (Figure 4A). CAGGCre-ER^{TM} line (Figure 4A line 1) can be used to excise DNA sequence flanked by two loxP sites (Figure 4A line 2). Excision in an embryo can be accomplished by injection of TM into a pregnant mother that carries double transgenic embryo for lines 1 and 2 (Figure 4B). In postnatal animals, excision is achieved either ubiquitously by intraperitoneal injection of TM or within a specific target organ by local injection of 4OH-TM. TM also works well in terms of recombination efficiency in a dose-dependent manner (Figure 4C). This powerful Cre system will be a very useful tool to modulate gene activity in embryos, adults, and culture systems where temporal control is an important consideration.

MORE mice: a germ line deleter : It is important to note that when we tried to make a transgenic line using the ROSA26 locus knock-in system, the gene that we expected to be over-expressed was not being expressed without cross-mating with Cre reporter line. This indicates that some of the reporters can be used as a germ line deleter for the constitutive expression of the gene. MORE (Mox2Cre) mice (Tallquist and Soriano, 2000) have Cre recombinase expression site from the Mox2 locus (Figure 5A). This locus drives the expression of Cre throughout the epiblast following implantation (Figure 5B-D). There has been greater than 95% deletion efficiency of the loxP flanked DNA in the germ line using the R26R strain as readout. Another germ line deleter is EIIa-Cre mice which controls Cre expression through the adenovirus EIIa promoter. It is also reported that internal loxP sites are deleted efficiently from all tissues tested, including the germ cells (Lakso et al., 1996).

Other site specific recombinase: combining with Cre/loxP system : The recombinase system most commonly used for conditional alleles in mouse is Cre/loxP (Branda and Dymecki, 2004; Wu et al., 2007) which is derived from

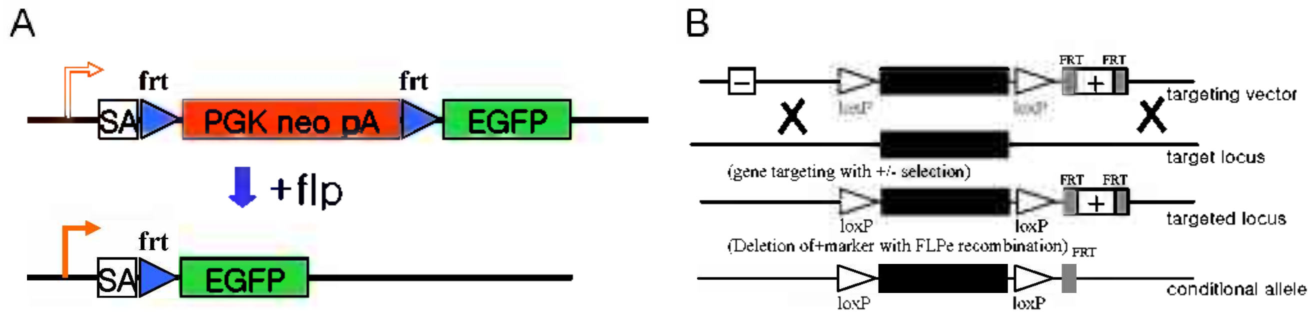

Figure 6. Usage of Flp/FRT recombination. (A) Overview of Flp/FRT recombination system. (B) Dual recombinase strategy for generating conditional gene targeting allele without positive selection marker.

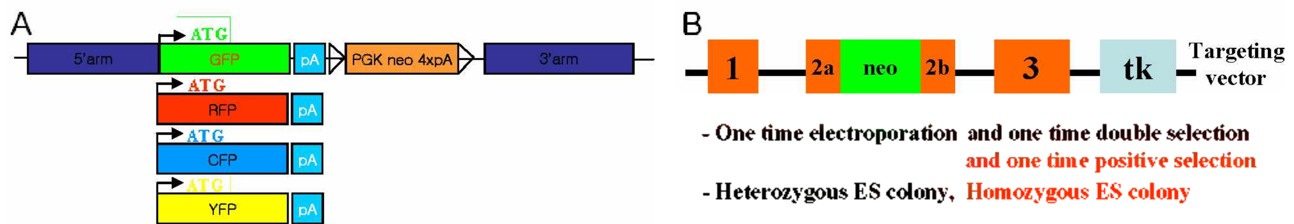

Figure 7. Considerations to produce null mutant mice. (A) Vector design for ATG-replacing reporter knock-in gene knock-out system. (B) Production of homozygous ES cells' colony with a single targeting.

bacteriophage P1 (Sauer and Henderson, 1988). While less utilized, the FLP/FRT system derived from *S. cerevisiae* (O'Gorman et al., 1991) has been markedly improved by Stewart and colleagues who cleverly used cycling mutagenesis to select for variant alleles of Flp that carry out recombination at high efficiency in mammalian cells (Figure 6A) (Wang et al., 2007). Sequential inactivation and reactivation of a given gene and testing the epistatic relationships between genes in somatic cells are now possible through a combination of Cre with the enhanced Flpe (Figure 6B). Recently, *Φ*C31 site specific recombinase from *Streptomyces lividans* has also been shown to possess a unique function in mammalian system (Belteki et al., 2003) which recombines between two heterotypic sites: *attB* and *attP*. An appropriate combination of these recombinases would improve the overall ability to characterize a gene function more precisely (Raymond and Soriano, 2007).

Loss-of-function study

To make null mutant mice, it takes at least about one year from the construction of the targeting vector if there is no delay during the whole procedure from steps such as colony selection of ES cells, chimera production and confirmation of germ line transmission for the mice. But, unfortunately, lots of cases are stuck in the chimera production as well as colony picking steps. Another limitation is the passage of the mutant genome from the chimera mice to their progeny. The actual time taken for the production of null mutant mice varies and it is usually needs more than what was expected and so as the cost involved. There is another big hurdle in terms of phenotype. There is no statistical report on how many researchers getting some phenotypes from their mutant mice. In most cases, no phenotypic differences can be found between the wild-type and the null mutant mice, even though their developmental cost and time are high. Very few cases actually succeeded in revealing the function of the expected gene based on the phenotype. This explains why this area is so hard to approach and therefore only a few research groups are pursuing it. A few strategic approaches that could help overcome the problems in this field are presented in the following sections.

Reporter knock-in gene knock-out system : There are limitations to understand the gene function with null mutant mice even when it has severe phenotypes. One of the ways to solve this problem is the usage of general reporter genes such as LacZ or fluorescence proteins to trace the gene expression. IRES (internal ribosomal entering site) or SA (splicing acceptor) are usually used for the efficient gene expression of the reporter, but they show inconsistent efficiencies among lines. As shown in Figure 7A, we developed a common vector in which the gene's start codon (ATG) was replaced with the reporter's, based on the concept that the entire gene has the same start codon. It may be the most reasonable way for tracing gene expression. In addition, it may give us consistency in terms of the level of gene expression between mouse lines that have different

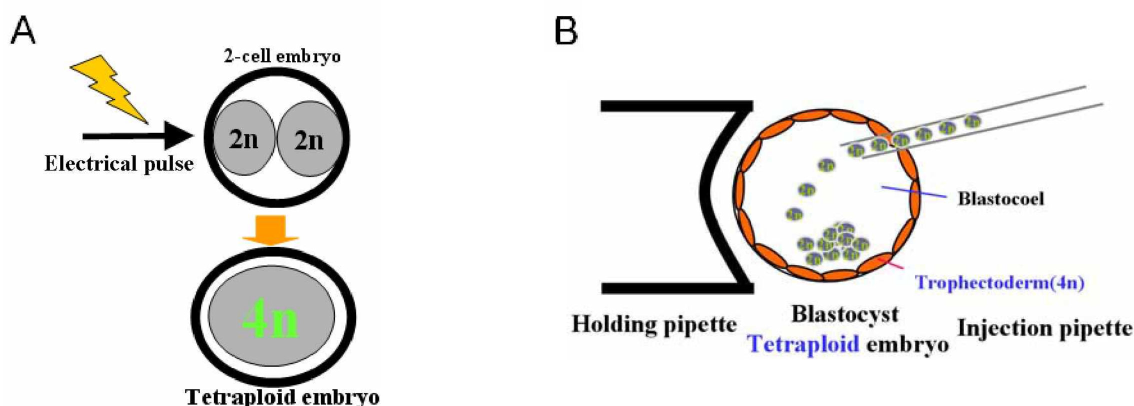

Figure 8. Homozygous mutant mice production. (A) Basic principle of tetraploid embryo formation. (B) Schematic diagram to produce gene manipulated mice using tetraploid embryo.

reporter systems (Figure 7A).

Homozygous mutant ES cells from a single targeting vector : The production of null mutants by homologous recombination, in which two copies of the gene have been inactivated by double targeting by two different positive selection markers, has been accomplished in ES cells (Riele et al., 1990; Deng et al., 1997). Such mutants allow one to investigate the function of a particular gene more easily in cell lines rather than in whole animals. More recently, an improved method was reported for obtaining such null mutant ES cells (Mortensen et al., 1992; Gorivodsky and Lonai, 2003) using single targeting construct. As shown in Figure 7B, it needs an extra one time positive selection with high concentrations of G418. There are several reports that homozygous cells have been known to be spontaneously produced from heterozygous cultured cells by a variety of mechanisms including chromosomal loss, gene deletion, mitotic recombination, gene conversion, or homologous recombination with episomally maintained construct DNA. Based on this hypothesis (spontaneous loss of heterozygosity), null mutant ES cells can be obtained with high G418 media from the heterozygous ES cells.

Mutant mouse production with tetraploid embryos

It is reported that tetraploid embryos can be implanted at high frequency but rarely form embryonic structures (Snow, 1975), whereas occasional embryos proceed through organogenesis and reached term (Snow, 1973). If tetraploid embryos at the blastomere stage are aggregated with diploid embryos, the tetraploid cells are selected during development of fetal tissues in most cases and persisted in extra-embryonal membranes (Tarkowski et al., 1977). Based on this concept, it is reasoned that the tetraploid cells might be complementary to the deficient extra-embryonal differentiation of ES cells, while they allow full expression for fetal development. Production of live fetuses that were entirely ES cells in genotype was achieved from such aggregates, showing that ES cells have the potential to form all fetal cell lineages (George et al., 2007). So, if the ES cells which have a null mutation in the genome aggregate with tetraploid embryos, null mutation pups can be produced directly without passing through the chimera selection and germ line transmission steps (Figure 8).

CONCLUSION

We briefly introduced recent progress in the gene manipulating system in mice in conjunction with on-going trials in our laboratories. This is no more than a spot in this field which is being widened in scope and in-depth rapidly in terms of application of diversity, efficiency of answering the questions, systemic approaches with developing tools, and so on. With these advancements, we would get an entire pool of gene-manipulated mice in the near future (Austin et al., 2004; Collins et al., 2007). It is also a challenging but exciting issue that well-equipped mouse system can be applied in other model systems such as zebra fish, *Drosophila melanogaster* and *Caenorhabditis elegans* to understand the systemic network of the whole body. It is also our hope that the technologies we described here will be expanded further to domestic animals and non-human primates (Hwang et al., 2006; Chrenek et al., 2007).

ACKNOWLEDGMENTS

This work was supported by a grant (Code # 20070401034031) from BioGreen 21 Program, Rural Development Administration, Republic of Korea and by a faculty research grant (2006-0047) of Yonsei University College of Medicine.

REFERENCE

Austin, C. P., J. F. Battey, A. Bradley, M. Bucan, M. Capecchi and

F. S. Collins. 2004. The knockout mouse project. Nat. Genet. 36:921-924.

Belteki, G., J. Haigh, N. Kabacs, K. Haigh, K. Sison, F. Costantini, J. Whitsett, S. E. Quaggin and A. Nagy. 2005. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. Nucleic Acids Res. 33:e51.

Belteki, G., M. Gertsenstein, D. W. Ow and A. Nagy. 2003. Site-specific cassette exchange and germline transmission with mouse ES cells expressing phiC31 integrase. Nat. Biotech. 21:321-324.

Branda, C. S. and S. M. Dymecki. 2004. Talking about a revolution: the impact of site-specific recombinases on genetic analyses in mice. Dev. Cell 6:7-28.

Capecchi, M. R. 2005. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat. Rev. Genet. 6:507-512.

Chrenek, P., L. Chrastinova, K. Kirchnerova, A. V. Makarevich and V. Foltys. 2007. The yield and composition of milk from transgenic rabbits. Asian-Aust. J. Anim. Sci. 20:482-486.

Collins, F. S., J. Rossant and W. Wurst. 2007. A Mouse for all reasons. Cell 128:9-13.

Deng, C., M. Bedford, C. Li, X. Xu, X. Yang, J. Dunmore and P. Leder. 1997. Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. Dev. Biol. 186:42-54.

Evans, M. J. and M. H. Kaufman. 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156.

George, S. H. L., M. Gertsenstein, K. Vintersten, E. Korets-Smith, J. Murphy, M. E. Stevens, J. J. Haigh and A. Nagy. 2007. Developmental and adult phenotyping directly from mutant embryonic stem cells. Proc. Natl. Acad. Sci. USA 104:4455-4460.

Gorivodsky, M. and P. Lonai. 2003. Novel roles of Fgfr2 in AER differentiation and positioning of the dorsoventral limb interface. Development 130:5471-5479.

Grippo, P. J., P. S. Nowlin, R. D. Cassaday and E. P. Sandgren. 2002. Cell-specific transgene expression from a widely transcribed promoter using Cre/lox in mice. Genesis 32:277-286.

Gu, X., C. Li, W. Wei, V. Lo, S. Gong, S-H. Li, T. Iwasato, S. Itohara, X-J. Li, I. Mody, N. Heintz and X. W. Yang. 2005. Pathological cell-cell interactions elicited by a neuropathogenic form of mutant Huntington contribute to cortical pathogenesis in HD mice. Neuron 46:433-444.

Hayashi, S. and A. P. McMahon. 2002. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev. Biol. 244:305-318.

Hitz, C., W. Wurst and R. Kuhn. 2007. Conditional brain-specific knockdown of MAPK using Cre/loxP regulated RNA interference. Nucleic Acids Res. 35:e90.

Hudziak, R. M., F. A. Laski, U. L. RajBhandary, P. A. Sharp and M. R. Capecchi. 1982. Establishment of mammalian cell lines containing multiple nonsense mutations and functional suppressor tRNA genes. Cell 31:137-146.

Hwang, S., E. J. Choi, S. You, Y. J. Choi, K. S. Min and J. T. Yoon. 2006. Development of bovine nuclear transfer embryos using life-span extended donor cells transfected with foreign gene. Asian-Aust. J. Anim. Sci. 19:1574-1579.

Ivanova, A., M. Signore, N. Caro, N. D. E. Greene, A. J. Copp and J. P. Martinez-Barbera. 2005. *In vivo* genetic ablation by Cre-mediated expression of dphtheria toxin fragment A. Genesis 43:129-135.

Jeong, J., J. Mao, T. Tenzen, A. H. Kottmann and A. P. McMahon. 2006. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordial. Genes Dev. 18:937-951.

Kisseberth, W. C., N. T. Brettingen, J. K. Lohse and E. P. Sandgren. 1999. Ubiquitous expression of marker transgenes in mice and rats. Dev. Biol. 214:128-138.

Koller, B. H. and O. Smithies. 1989. Inactivating the beta 2-microglobulin locus in mouse embryonic stem cells by homologous recombination. Proc. Natl. Acad. Sci. USA. 86:8932-8935.

Lakso, M., J. G. Pichel, J. R. Gorman, B. Sauer, Y. Okamoto, E. Lee, F. W. Alt and H. Westphal. 1996. Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. Proc. Natl. Acad. Sci. USA. 93:5860-5865.

Mao, A., B. Barrow, J. McMahon, J. Vaughan and A. P. McMahon. 2005. An ES cell system for rapid, spatial and temporal analysis of gene function *in vitro* and *in vivo*. Nucleic Acids Res. 33:e155.

Mortensen, R. M., D. A. Conner, S. Chao, A. A. T. Geisterfer-Lowrance and J. G. Seidman. 1992. Production of homozygous mutant ES cells with a single targeting construct. Mol. Cell. Biol. 12:2391-2395.

Murtaugh, L. C., B. Z. Stanger, K. M. Kwan and D. A. Melton. 2003. Notch signaling controls multiple steps of pancreatin differentiation. Proc. Natl. Acad. Sci. USA. 100:14920-14925.

O'Gorman, S., D. T. Fox and G. M. Wahl. 1991. Recombinase-mediated gene activation and site-specific integration in mammalian cells. Sci. 251:1351-1355.

Odorfer, Ko. I., N. J. Unger, K. Weber, E. P. Sandgren and R. G. Erben. 2007. Marker tolerant, immunocompetent animal as a new tool for regenerative medicine and long-term cell tracking. BMC Biotech. 7:30.

Raymond, C. S. and P. Soriano. 2007. High-efficiency FLP and ΦC31 site-specific recombination in mammalian cells. PLoS ONE 2:e162.

Riele, T., E. R. Maandag, A. Clarke, M. Hooper and A. Berns. 1990. Consecutive inactivation of both alleles of the pim-1 proto-oncogene by homologous recombination in embryonic stem cells. Nature 348:649-651.

Sauer, B. and N. Henderson. 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc. Natl. Acad. Sci. USA. 85:5166-5170.

Snow, M. H. 1973. Tetraploid mouse embryos produced by cytochalasin B during cleavage. Nature 244:513-515.

Snow, M. H. 1975. Embryonic development of tetraploid mice during the second half of gestation. J. Embryol. Exp. Morph. 35:81-86.

Soriano, P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21:70-71.

Srinivas, S., T. Watanabe, C-S. Lin, C. M. William, Y. Tanabe, T. M. Jessell and F. Costantini. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the

ROSA26 locus. BMC Dev. Biol. 1:4.

Tallquist, M. D. and P. Soriano. 2000. Epiblast-restricted Cre expression in MORE mice: A tool to distinguish embryonic vs. extra-embryonic gene function. Genesis 26:113-115.

Tarkowski, A. K., A. Witkowska and J. Opas. 1977. Development of cytochalasin B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. J. Embryol. Exp. Morph. 41:47-64.

Wang, W., M. Warren and A. Bradley. 2007. Induced mitotic recombination of p53 *in vivo*. Proc. Natl. Acad. Sci. USA. 104:4501-4505.

Wu, S., G. Ying, Q. Wu and M. R. Capecchi. 2007. Toward simpler and faster genome-wide mutagenesis in mice. Nat. Genet. 39:922-930.

Yu, H-M., B. Liu, S-Y. Chiu, F. Costantini and W. Hsu. 2005. Development of a unique system for spatiotemporal and lineage-specific gene expression in mice. Proc. Natl. Acad. Sci. USA. 102:8615-8620.

Yu, J. and A. P. McMahon. 2006. Reproducible and inducible knockdown of gene expression in mice. Genesis 44:252-261.

Zambrowicz, B. P., A. Imanoto, S. Fiering, L. A. Herzenberg, W. G. Kerr and P. Soriano. 1997. Disruption of overlapping transcripts in the ROSA βgeo26 gene trap strain leads to widespread expression of β-galactosidase in mouse embryos and hematopoietic cells. Proc. Natl. Acad. Sci. USA. 94:3789-3794.