ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2007, **39**(4): 235–246 **CN** 31-1940/Q

Minireview

Tetracycline-inducible Expression Systems: New Strategies and Practices in the Transgenic Mouse Modeling

Yan SUN[1,2], Xigu CHEN[1]*, and Dong XIAO[2]*

[1] *Center of Experimental Animals, Sun Yat-Sen University, Guangzhou 510080, China;*
[2] *Institute of Comparative Medicine and Center of Experimental Animals, Southern Medical University, Guangzhou 510515, China*

Abstract To accurately analyze the function of transgene(s) of interest in transgenic mice, and to generate credible transgenic animal models for multifarious human diseases to precisely mimic human disease states, it is critical to tightly regulate gene expression in the animals in a conditional manner. The ability to turn gene expression on or off in the restricted cells or tissues at specific time permits unprecedented flexibility in dissecting gene functions in health and disease. Pioneering studies in conditional transgene expression have brought about the development of a wide variety of controlled gene expression systems, which meet this criterion. Among them, the tetracycline-controlled expression systems (e.g. Tet-off system and Tet-on system) have been used extensively *in vitro* and *in vivo*. In recent years, some strategies derived from tetracycline-inducible system alone, as well as the combined use of Tet-based systems and Cre/*lox* P switching gene expression system, have been newly developed to allow more flexibility for exploring gene functions in health and disease, and produce credible transgenic animal models for various human diseases. In this review these newly developed strategies are discussed.

Key words tetracycline-inducible expression system; Cre/*lox* P system; transgenic mouse modeling; leaky expression; stringent control; lineage-specific gene expression; lineage-specific RNAi

The Human Genome Project has revealed the sequence information (http://wit.integratedgenomics.com/GOLD) of many of the genes that make us what we are. Therefore, a significant challenge for scientists over the next few decades is to annotate the human genome with functional information. This effort will enable us to gain a full understanding of the molecular mechanisms and pathways underlying normal development, as well as those responsible for pathogenesis.

One powerful approach is the transgene overexpression of any given gene(s) in genetically engineered mouse model to explore the role(s) of the gene(s) *in vivo*. Conditional transgenic mice are becoming increasingly popular for precisely regulate gene expression in a temporal and spatial pattern. The ideal controlled over-expression system should permit the investigators to rapidly and reversibly switch the transgene expression on and off, exclusively in the desired cells or tissue(s) at any time point during development. Tetracycline (Tet)-inducible expression system is one of the most prominent and widely-accepted inducible systems so far. There are two basic variants of the tetracycline-inducible expression system: the tTA (Tet-off) system [1] and the rtTA (Tet-on) system [2]. These systems, especially Tet-on system, have been most extensively employed in the transgenic mouse modeling [3–7].

The tetracycline-dependent regulatory systems (Tet-based systems) have been successfully employed to define the development-dependent and development-independent

Received: October 9, 2006 Accepted: December 11, 2006
This work was supported by the grants from the National Natural Science Foundation of China (No. 30271177 and 39870676), the National 9th Five-year Program (No. 101033), the Major Science and Technology Projects of Guangdong Province (No. B602), the Natural Science Foundation of Guangdong Province (No. 021903), the Postdoctoral Fellowship Foundation of China (Series 29), and the Special Fund of Scientific Instrument Collaborative Share-net in Guangzhou (No. 2006176)
*Corresponding authors:
Dong XIAO: Tel, 86-20-62789009; E-mail, Xiao_d@hotmail.com
Xigu CHEN: Tel, 86-20-33151566; E-mail, Xiguchen@163.com

DOI: 10.1111/j.1745-7270.2007.00258.x

biological and pathological processes [4–7]. These systems, however, are not always tight but leaky because of the inherent defects in Tet-based systems and promoter leakiness [5,6,8–12]. In some cases, leak is not a big problem; in some others, however, it is a real problem and must be avoided [9–13].

By integrating the tetracycline-dependent expression systems with an Cre/*lox* P switching gene expression system, four labs [14–17] have already developed a unique transgenic mouse system based on (r)tTA dependent doxycycline (Dox)-mediated activation of target gene(s) following a Cre-excision event to achieve a temporal, spatial and lineage-specific gene expression in mice. This unique and versatile system is very useful for functional analyses within specific cells or tissue(s) with the added advantage of temporal regulation of gene activity within daughter cells of a specific lineage in an inducible manner.

This review will give a brief introduction of the tetracycline-dependent expression systems and focus on the newly developed or developing strategies and their applications in transgenic mouse modeling.

Components and Working Principles of Tet-based Systems and Cre/*lox* P System

Tetracycline-inducible expression systems

Together with a specific promoter, the tetracycline-inducible systems permit relatively stringent, reversible (on↔off), quantitative, temporal and spatial regulation of transgene expression in a wide range of cells in culture [8], as well as in transgenic animals (http://www.zmg.uni-mainz.de/tetmouse) [3–7]. Use of the Tet-based systems requires two coordinate building blocks: the ligand-dependent transactivator (r)tTA as the effector and a *tetO*-CMV minimal promoter cassette regulating the expression of the transgene as the responder. **Fig. 1(A)** fully demonstrates the underlying mechanisms of the Tet-on system in the transgenic mouse modeling.

Cre/*lox* P switching expression system

Currently, the most widely used site-specific DNA recombination system in mice is Cre/*lox* P system [18, 19]. The Cre/*lox* P system has two components: Cre recombinase derived from bacteriophage P1 and two 34-bp *lox* P sites that Cre recognizes; the site-specific recombination is accomplished by Cre-mediated catalysis of reciprocal recombination between the two *lox* P sites in both tissue culture cells and mice [18,19]. The general strategy for conditional gene expression mediated by the Cre/*lox* P switching expression system is to excise the intervening transcription STOP cassette [**Fig. 1(B)**] or DNA sequence flanked by two *lox* P sites placed in the same direction, thereafter achieving conditional gene activation [**Fig. 1(B)**] or inactivation [18,19]. The conditional regulation of transgene expression is accomplished by operating Cre expression in a specific place and/or at a specific time.

Optimal "off/on" Regulation of Transgene Expression Accomplished by the Improved Tet-based Systems in the Transgenic Mouse Modeling

Leaky expression in Tet-based systems compromises the expected tight regulation

If a gene is to be kept inactive most of the time and turned on only occasionally, Tet-on system appears to be more appropriate than Tet-off system; moreover, of these two systems, rtTA system is more suitable for rapid induction of gene expression.

But unfortunately, leaky expression, which is derived from both the inherent defects in Tet-based systems and the promoter leakiness caused by promoter-dependent or integration site-dependent effects, compromises the desired stringent regulation of transgene expression [5,6,8–13]. In theory, the promoter leakiness may be effectively avoided if large numbers of transgenic microinjections are undertaken to make a great number of transgenic animals. The inherent shortcomings are attributed to the fact that rtTA retains some affinity for *tetO* sequences even without Dox and to the unwanted residual activity of the *tetO*-CMV responder even when the active (r)tTA is absent [**Fig. 1(A)**] [5,6,9–11]. This is evident from the undetectable or detectable expression levels of transgene activation, and further phenotype induction in animals or cells which are not receiving Dox [5,6,9–13].

Under most circumstances, the leak in Tet-based systems is quite acceptable by the investigators who use these systems. In some circumstances, however, the detectable and undetectable levels of undesired transgene leaky expression in animals greatly limits the use of Tet-based systems in precisely modeling the complex biological or human disease processes or in evaluating the effects of gene product(s).

For instance, if the product(s) of the given transgene(s) is/are toxic or unwanted, even a low level of expression could be detrimental to embryos, preventing any further

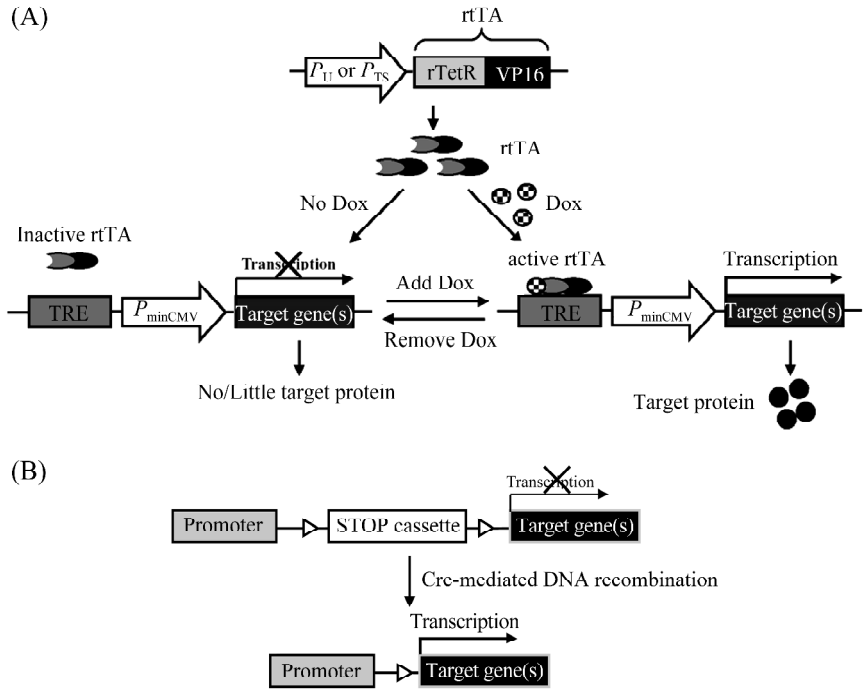

Fig. 1 General strategies for conditional expression of target gene(s) mediated by Tet-on system or Cre/*lox* P switching expression system in transgenic mouse modeling

(A) Reverse Tet-controlled transcriptional activator (rtTA) system: "Tet-on" system. Tet-based systems can be used to conditionally activate gene expression in mice. *In vivo*, the systems generally require two independent types of transgenic mice to be generated, *i.e.*, transactivator mice, in which regulatory protein rtTA is constitutively produced under the control of a ubiquitous promoter (P_U) or tissue- or cell type-specific promoter (P_{TS}), and responder mice, in which the expression of cDNA(s) of interest is under the regulation of the rtTA-dependent promoter. Breeding generates double-transgenic mice. In double transgenic mice, addition of Dox to Tet-on system results in transcriptional induction of the gene(s) of interest, whereas in the absence of Dox, inactive rtTA cannot bind its target or dissociates from TRE, and subsequently terminates the transcription of downstream target gene(s). Addition of Dox to or withdrawal of Dox from drinking water of the bi-transgenic mice allows to *in vivo* modulate the expression of any given gene(s) in a quantitative, time-controlled and ubiquitous or tissue/cell type-specific manner. In Tet-based inducible systems, tissue or cell-type specificity of the expression of any given gene(s) is conferred by the promoter driving rtTA expression. (B) Cre/*lox* P switching expression system. To realize Cre-mediated gene activation, a *lox* P-flanked intervening transcription STOP cassette sequence (SCS) [18,19] is inserted between the promoter and the coding region for the gene(s) of interest. In the absence of Cre, the expression of target gene(s) is completely prevented by STOP sequence flanked by *lox* P sites, while in the presence of Cre, Cre-mediated DNA recombination results in removal of STOP cassette, followed by activating the expression of any given gene(s) under investigation. In Cre/*lox* P switching expression system, tissue or cell-type specificity of the expression of target gene(s) is conferred by the promoter driving the expression of Cre gene or the purposed gene(s). Tet-on System rtTA ( ) binds TRE and activates transcription in the presence of Dox. ▷, *lox* P site.

analysis of the potential phenotype during late antenatal development or in the infancy and adult. Tet-off system was employed in the transgenic mouse modeling to govern the regulated expression of the cell-autonomous, lethal diphtheria toxin A (DTA) gene [20]. Data indicated that transgenic mice (responder mice), which harbored the *tetO*-DTA target transgene, were generated at a ten-fold lower frequency compared with previous production [20].

The complete "off" and "on" regulation derived from the tightly controlled gene expression system will be very useful to express the antigen genes of hepatitis virus in a temporally restricted fashion and precisely define the immunological reactions against transgene products such as infectious agents and pathogenesis of hepatitis. When the activation of target gene expression in the animals is mediated by the Cre/*lox* P switching expression system, the transgenic animal is immunocompetent for the transgene product(s). To examine the immune response to hepatitis C virus (HCV) structural proteins and pathogenesis of hepatitis C, Wakita *et al*. [21] used the Cre/*lox* P system to express the core, E1 and E2 proteins efficiently and conditionally in transgenic mice (e.g. CN2 mice), providing a useful "non-immune tolerant" animal model with which to investigate the host immune response against HCV infection and the pathogenesis of hepatitis C [21–24]. The hepatitis

B virus (HBV) and HCV transgenic animals derived from non-tight gene expression regulatory systems (i.e., heavy metal-inducible MT-1 promoter with high basal activity in the absence of induction [25–27], Tet-off system [12] and Tet-on system [10]) and from constitutive gene expression systems [10] are not immunocompetent for the transgene product(s), e.g. viral antigen(s), because the viral antigen gene(s) begin(s) to express before the formation of immune system of organism. In these "immune tolerant" mice, the immune system can not recognize the xenobiotic nature of these viral antigen(s); actually, the immune system plays rather important roles in the pathogenesis of both hepatitis B and C [12,21–24]. Although Tet-based systems permit relatively tight regulation of transgene expression and leaks very slightly, the data demonstrated that the amount of leakage was not enough to detect biochemically but sufficient to induce immune tolerance [12].

The phenotypes and pathological analysis demonstrates that CN2 mice for HCV, but not "immune tolerant" animal model for HBV or HCV can mimic disease states in human precisely and truly. In "non-immune tolerant" CN2 mice, several days after the transgene activation, the core, E1 and E2 proteins could be detected in liver lysates with concurrent increases in serum alanine aminotransferase levels, and the level of circulating core protein was primarily dependent on hepatocyte destruction; subsequently, seven days and fourteen days after AxCANCre administration, the development of substantial hepatic pathology in CN2 mice which was not present in the naive CN2 mice or CD41&CD81 cell-depleted CN2 mice was revealed in the liver and the anti-core antibody response was detected, respectively [21–24]. The findings from CN2 mice suggest that HCV proteins are not cytopathic directly and that the host immune response plays an important role in HCV infection. Whereas in "immune tolerant" transgenic mice for HBV and HCV, the expression of hepatitis virus antigen genes was detected in the liver, but there have no significant differences between "immune tolerant" transgenic mice and normal mice in serum ALT and aspartate aminotransferase levels, the antibodies against virus antigens in serum were not detected, and the normally pathological changes in the liver could not be observed [12,25–31]. Collectively, the "immune tolerant" animal models for HBV or HCV derived from non-stringent gene expression regulatory systems, which may not allow for the development of an ideal disease model for human hepatitis, and from the constitutive gene expression systems can not precisely and truly mimic the states of human diseases, such as virosis hepatitis.

Although the tetracycline-inducible systems with acceptable levels of leak have been used successfully to define the development-dependent and -independent biological and pathological processes, to some extent, the confounding effects of transgene leak in Tet-based systems may interfere with analysis of gene functions and phenotype in the transgenic mouse modeling. Combined tTS and rtTA systems, but not rtTA system alone, can be used to define the natural process of *in vivo* injury and repair responses accurately and truly [11,13]. The incorporation of tTS into Tet-based transgenic system effectively decreased basal transgene leak to undetectable levels and totally eliminated the IL-13-induced phenotype without Dox administration [13]. Furthermore, in the CC10-rtTA-IL-13 double transgenic mice, IL-13, mucus metaplasia, inflammation, alveolar enlargement and enhanced lung volumes were noted at base line, and increased greatly after Dox administration, whereas in the CC10-rtTA/tTS-IL-13 triple transgenic animals, IL-13 and the IL-13-induced phenotype could not be achieved without Dox [13]. The combined use of tTS and rtTA systems in producing transgenic mouse models with a truly regulatable "on/off" switch of gene expression is very useful in studies in which the critical windows of development and the natural process of injury and repair are defined precisely and truly.

Taken together, although Tet-based inducible transgenic modeling systems with acceptable levels of leak have provided a great deal of valuable information on the functions of many genes and on the processes and pathogenesis of some human diseases, under certain conditions such "leaky" systems derived from intrinsic defects of systems are not always tolerable for models of more complex human diseases and also not suitable for more accurately answering the complex questions: for example, whether the effects of transgene products are development-dependent?

Uses of improved rtTA variants, tTS and pTRE-Tight vector in transgenic mouse modeling

Though Tet-based systems have defect, i.e. basal transgene leak derived from inherent defects in Tet-based systems *in vitro* and *in vivo*, increasing evidences showed that a more tightly controlled regulatory system can be readily achievable *in vitro* [32–39], *ev vivo* [37,38] and *in vivo* [13,40–44] when the advanced versions (e.g. rtTA2^S-M2 and rtTA2^S-S2) of rtTA [45], tetracycline-controlled transcriptional silencer (tTS) [46] and an "ideal" minimal promoter in responsive components (pTRE-Tight) (http://www.bdbiosciences.com/clontech/techinfo/vectors/vectorsT-Z/pTRE-Tight.shtml) are employed alone or in combination therein. For instance, more stringent control

of transgene expression using improved versions of rtTA, for example rtTA2^S-S2 [40,41] and rtTA2^S-M2 [17,42,43], has also been achieved in transgenic mice for Cre recombinase [40], ferritin H [41], pigment epithelium-derived factor (PEDF) [42] and lac Z [17,43]. tTS can efficiently reduce or completely eliminates background expression in transgenic mice by combining tTS with rtTA [13]. Information on the countermeasures available to eliminate basal transgene leak of Tet-based systems has recently been summarized [10]. We suppose that these properties would also allow the generation of transgenic mice with pre-selected expression windows.

Combinatorial use of tTS system and Tet-on advanced system in transgenic mouse modeling requires the generation of another transgenic strain harboring the tTS transgene under the control of a ubiquitous promoter or a cell-type/tissue-specific promoter. tTS transgenic mice are crossed with bi-transgenic mice (e.g. rtTA-advanced/target gene mice) to produce triple transgenic offspring, allowing more tight control of transgene expression. Although an obvious disadvantage of this approach is the need to generate triple transgenic animals, there is the alternative of combining the two control elements (i.e. rtTA-advanced and tTS) in a single transgene under the control of a ubiquitous promoter or a cell-type or tissue-specific promoter to produce rtTA-IRES-tTS transgenic mice (**Fig. 2**). This "two-in-one" and "all-in-one" strategy have been confirmed *in vitro* and *ev vivo* [32–34,37,38].

General strategies for more stringent control of transgene expression by the improved Tet-on system in transgenic mouse modeling are fully presented in **Fig. 2**. The strategy of crossing effector & silencer transgenic mice and acceptor transgenic mice is more convenient and flexible because researchers are entitled to make full use of a huge database of the different ubiquitous or cell/tissue-specific rtTA or rtTA-advanced-tTS lines (http://www.zmg.unimainz.de/tetmouse) to examine the transgene effects. At present, investigators are required to produce the acceptor transgenic mice harboring gene(s) of interest, and subsequently mate them with a selected effector and silencer from the database to attain bi-transgenic lines. In a word, the developed and developing cell-type/tissue-specific rtTA-advanced-tTS transgenic lines can be combined with other transgenic responder lines for more tight and cell-type/tissue-specific over-expression of any target gene(s).

Co-injection strategy Furthermore, using one transgene system in which the tTS construct, rtTA-advanced construct and TRE$_{mod}$-transgene construct are all co-microinjected or integrated in a single transgenic construct

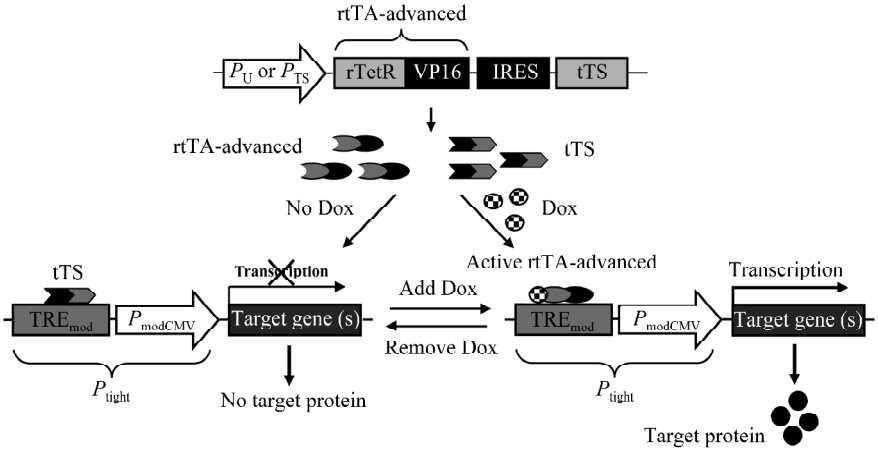

Fig. 2 General strategy for more tight regulation of target gene expression by Tet-on advanced system in transgenic mouse modeling

In transgenic mouse modeling, the advanced system require two independent types of transgenic mice to be generated, i.e., transactivator and transcriptional silencer mice, in which rtTA-advanced (including rtTA2^S-S2 and rtTA2^S-M2) and tTS are constitutively produced under the control of a ubiquitous promoter (P_U) or tissue- or cell type-specific promoter (P_{TS}), and responder mice, in which expression of cDNA(s) of interest is under the regulation of the rtTA-dependent P_{tight} promoter. Breeding produces double transgenic mice. In double transgenic mice, in the absence of Dox, tTS, which is similar in function to that of tTA, binds TRE, and thereby actively silences the transcription of the downstream target gene(s); whereas a sufficient concentration of Dox is present, tTS dissociates from *tetO*, relieving the transcriptional suppression and allowing active improved versions of rtTA, such as rtTA2^S-S2 and rtTA2^S-M2, to bind to *tetO* sequences, which then activate transcription of the gene(s) under investigation. To avoid crossing the three transgenic lines to obtain triple transgenic mice, rtTA-advanced and tTS are combined on a single DNA molecule, which is employed to produce the transgenic mice. rtTA-advanced (▰) binds TRE and activates transcription in the presence of Dox, tTS (▰) binds TRE and suppresses transcription in the absence of Dox. Other details as in **Fig. 1**.

is less time consuming, less labor consuming, and at the same time avoids the segregation of control elements during breeding. When two or several transgenic constructs are co-injected into single cell fertilized embryos, in general, the co-injected constructs are typically co-integrated into the same site(s) of the genome [47]; since transgenes often tend to insert into the genome in a head to tail pattern, this would provide transgene (+) animals with tight transgene control in which all constructs are passed to progeny as if they are a single gene. This would minimize the breeding and genotyping required for phenotypic analysis. For example, simultaneous microinjection of CC10-rtTA, CC10-tTS and tetO-TGF-b1 constructs successfully generated an inducible TGF-b1 mouse model in Elias's laboratory [11].

"All-in-one" strategy Some investigators integrated the two expression units of Tet-off system [48] or Tet-on system [49–51] on a single DNA fragment in transgenic mouse modeling, and demonstrated that this "two-in-one" system is functional in transgenic mice. In addition, a simple "all-in-one" vector, containing all of the elements of the Dox-inducible Tet-on system in their most advanced variants (rtTA2^S-M2 and tTS), can be used to control transgene expression efficiently in long-term tissue culture and in the mouse hematopoietic system following bone marrow transplantation [32]. Bornkamm *et al*. [33] and Epanchintsev *et al*. [34] achieved more stringent Dox-dependent control of gene activities and shRNAmir expression (see below for more details) *in vitro* using an episomal one-vector system (pRTS-1, a simple "all-in-one" vector), which carries all the elements (including rtTA2^S-M2, tTS and the bidirectional promoter P(tet)bi-1 in the same transgene construct), respectively. The vector of pRTS-1 was confirmed to be low background activity *in vitro* [33]. However, whether this "all-in-one" system is functional in transgenic mice remains to be fully elucidated. Anyway, in the first place, this "two-in-one" or "all-in-one" strategy eliminates the need to generate two independent transgenic lines and greatly facilitates the mouse breeding strategy as effector and responder will not segregate; in addition, integrating an equal number of target and transactivator genes may allow for a more accurately quantitative regulation of purposed gene expression.

Temporal, Spatial and Lineage-specific Gene Expression Accomplished by Integrating Tet-based Systems and Cre/*lox* P System

Two powerful systems, tetracycline-inducible systems and Cre/*lox* P system, are extensively and successfully employed in the transgenic mouse modeling; tetracycline-inducible systems allow a reversible/temporal regulation of transgene expression by addition of Dox to or withdrawal of Dox from drinking water, whereas Cre/*lox* P system permits gene(s) to be permanently activated or inactivated in the specific lineage at a specific time point [18,19]. Recently four individual labs [14–17] have already developed a unique and versatile transgenic mouse system based on (r)tTA dependent, Dox-mediated regulation of target gene(s) following a Cre-deletion event to realize temporal, spatial and lineage-specific gene expression in mice.

A triple, unique and versatile system developed in the two individual labs [14,17] is well demonstrated in **Fig. 3**. In this system, for the reliable rtTA expression in a broad range of cell types, rtTA-IRES-EGFP [14] or rtTA2^S-M2 [17] transgene, preceded by a *lox* P-flanked STOP sequence, is integrated into the ROSA26 (R26) locus to create a R26-STOP-rtTA-IRES-EGFP or R26-STOP-rtTA2^S-M2 transgenic mouse strain with the rtTA or rtTA2^S-M2 expression under the regulation of an endogenous and ubiquitous R26 promoter, respectively. Therefore, in this system, Cre-mediated deletion of STOP cassette from ROSA26 locus will immediately activate the rtTA [14] or rtTA2^S-M2 [17] expression in the Cre-expressing cells, and thereafter R26-rtTA gene activated by the excision of STOP cassette remains active in all of their derivatives regardless of Cre expression in these cells, in contrast to the Tet-based systems in which (r)tTA is expressed directly under the control of a ubiquitous promoter or a cell type or tissue-specific promoter (**Fig. 2**). Thus, the use of a proper Cre transgenic mouse line (available at http://www.mshri.on.ca/nagy) enables the cell type-, tissue-, or lineage-specific expression of rtTA, in other words, the promoter specificity of Cre transgene in mice determines where the Tet-based system becomes active. In the Cre-expressing cells and their daughter cells, the gene(s) under investigation is/are silent in the absence of Dox; while exposure to Dox, the active rtTA activates the expression of any given gene(s). Collectively, after Cre-mediated deletion of STOP cassette, the expression of target transgene is further controlled by Tet-on system in a temporal and lineage-specific manner. For example, the ability of this versatile system for targeted gene expression was primarily demonstrated in the neuroepithelial and hematopoietic lineages, and in the derivatives of neural crest and in the mammary epithelium [17] and in neural crest linage [14].

Moreover, other two labs [15,16] developed another

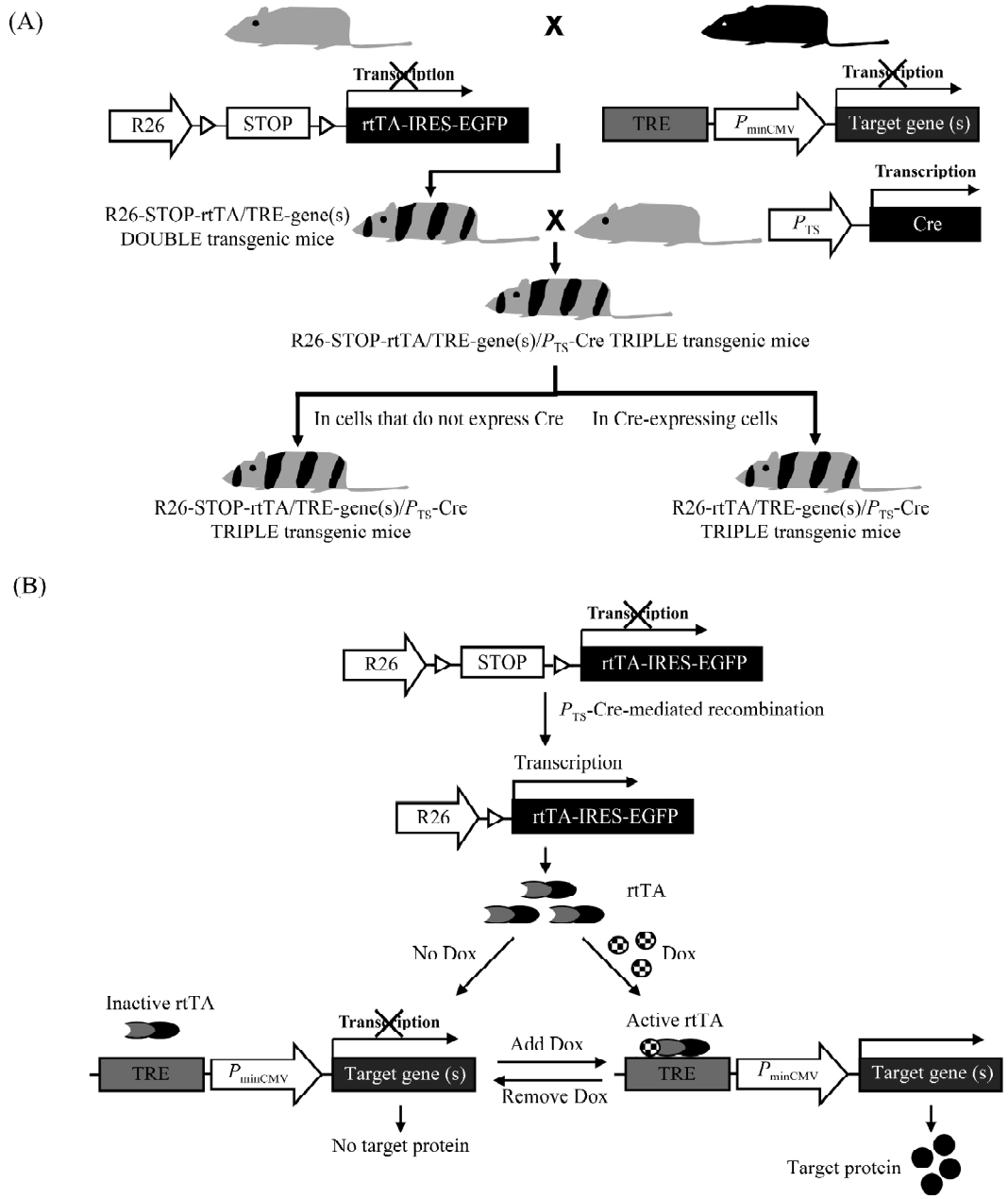

Fig. 3 Strategy for temporal, spatial and lineage-specific gene expression accomplished by combined use of Tet-on system and Cre/*lox* P system

(A) Mating strategy for generating R26-STOP-rtTA/TRE-gene(s)/P_{TS}-Cre TRIPLE transgenic mice. Firstly, transgenic mice which are homozygous for the targeted insertion of rtTA-IRES-EGFP transgene at R26 are bred with homozygous TRE-gene(s) transgenic mice, a Dox-inducible rtTA-dependent TRE-gene(s) responder line, to attain R26-STOP-rtTA/TRE-gene(s) double transgenic mice. Secondly, R26-STOP-rtTA/TRE-gene(s)/P_{TS}-Cre triple transgenic mice are generated by crossing R26-STOP-rtTA/TRE-gene(s) mice with any given P_{TS}-Cre transgenic mice which constitutively express Cre protein under the control of a tissue- or cell type-specific promoter (P_{TS}). This mating strategy is very useful to dissect the functions of any given gene(s) in the desired tissue or cell-type using a cell type- or tissue-specific Cre transgenic mouse strain. (B) Illustration for spatiotemporal and lineage-specific gene expression using a dual-level expression system. In this advanced system, tissue or cell-type and lineage specificity is conferred by Cre-mediated excision of the *lox* P-flanked STOP cassette in Cre-expressing cells, and further temporal activation of target gene(s) is controlled by Dox administration in the specific time point. In this dual-level expression system, rtTA-IRES-EGFP expression is independent of regulation by P_{TS} after STOP cassette excision, therefore, R26-rtTA gene activated by Cre-mediated DNA recombination remains active in all of the descendants derived from precursors even after Cre expression has been switched off. In cells which do not express Cre protein, both rtTA and EGFP are not produced, thereby, TRE-gene(s) is silent, whereas in Cre-expressing cells, rtTA and EGFP expression is switched on. Dox inducer is absent, inactive rtTA cannot activate the expression of target gene(s) under investigation, whereas any given gene(s) is/are expressed after Dox addition. In Cre-expressing cells, the deletion of STOP cassette permits rtTA and EGFP expression under the control of a ubiquitous and endogenous ROSA26 (R26) promoter in all of daughter cells derived from precursors. Other details as in **Figs. 1** and **2**. ▷, *lox* P site.

advanced and versatile system in which a Tet-off regulation unit as a single cassette was readily integrated into the ROSA26 locus of mouse embryonic stem cells (ES cells) and becomes active after Cre-mediated excision of STOP cassette in the Cre-expressing cells and their descendants. In the system developed by Miyazaki's lab [16], the tTA expression is conditional to a Cre-mediated excision of a STOP cassette (inserted between promoter and tTA gene) from the ROSA26 locus [16], whereas the other developed by McMahon's lab [15], a STOP cassette is inserted between P_{minCMV} promoter and target gene(s). Using the approach, the feasibility of dual regulatory drug control in the regulation of targeted allele by manipulating Hh signaling with a SmoM2-YFP allele was successfully tested in McMahon's lab [15]; in this lab, the established Cre-*lox* P and Dox regulated 3-1 ES cell line is being used to be spatio-temporal manipulation of each of five major development-related signaling pathways, such as Hedgehog, Wnt, BMP, FGF and Notch, to aid in genetically exploring the complex processes *in vitro* and *in vivo* [15].

The strategies and approaches presented here abrogates the need to produce and characterize new transgenic mouse lines expressing (r)tTA in the desired tissue or cell-type specific pattern where a large number of well-characterized cell-type or tissue-specific Cre transgenic mouse strains already exist in a voluntary database of Cre-expressing mice (http://www.mshri.on.ca/nagy). These unique and versatile systems will be useful for investigating genes, in which time-dependent regulation of transgene expression is pretty necessary for fully exploring the full spectrum of gene functions within descendants of a specific lineage with a great flexibility, which is pretty difficult to achieve with the conventional systems, such as Tet-based systems alone or Cre/*lox* P system only.

Reversible, Temporal and Spatial Gene Silencing by shRNAmir Expression in the Transgenic Mouse Modeling

The advent of RNA interference (RNAi) has led to the ability to revolutionize the functional analysis of genes *in vitro* and *in vivo*. The vector-based RNAi has already been used to successfully achieve the specific and stable knockdown of endogenous genes with high efficiency in the transgenic mice [52]. A number of vector-based Cre-controlled transgenic RNAi systems for conditional RNAi have been described previously [53–58]. Temporal and/or spatial knockdown of endogenous gene expression with high efficiency in mice can be reproducibly realized by conditionally controlling of siRNA expression using the Cre/*lox* P system [56–59]. Increasing data demonstrated that stable and efficient knockdown of endogenous gene expression in mice through vector-based transgenic RNAi strategy can generate phenotypes of gene knockout in mice [59–61]. Together, the vector-based transgenic RNAi strategy offers a technically simpler, cheaper and quicker alternative to classical gene knockout technology in deciphering gene functions *in vivo* in a temporal and/or spatial manner.

Although limited choices of RNA polymerase (Pol) III promoters have been developed to express shRNA, a large repertoire of Pol II promoters have been successfully used in transgenic mice. microRNAs (miRNAs), the endogenous form of shRNAs that carry out gene silencing function, are downstream of Pol II promoters and are expressed by Pol II activity [61,62]. microRNA-adapted shRNA (shRNAmir) construct, containing Pol II promoter driving the expression of an shRNA with a structure that mimics human miRNA miR-30a, has been successfully designed to mimic a natural miRNA primary transcript, enabling specific processing by the endogenous RNAi pathway and producing more effective and specific gene knockdown derived from microRNA-30 adapted design [61,62].

Recently, the expression arrest shRNAmir libraries, which were designed to facilitate the RNAi-mediated down-regulation of all human or mouse genes, were developed [62]. A large collection of human and mouse pSM2-based shRNAmir sequences are publicly available from Open Biosystems (http://www.openbiosystems.com), which can be easily transferred from pSM2c vector to the vector of interest by the ligation-free MAGIC technique [63].

Temporal, spatial and lineage-specific RNAi-mediated gene silencing accomplished by integrating Tet-based systems and Cre/*lox* P system

Recently, a series of lentiviral vectors (called pPRIME, potent RNAi using shRNAmir expression), which provide high penetrance Tet-regulatable knockdown of gene expression at single copy *in vitro*, are newly developed in Prof. ELLEDGE's lab [64]. If integrating this pPRIME system with RNAi regulated by Tet-based systems and the versatile system mentioned above [14,17], the temporal, spatial and lineage-specific loss of gene function in mice can be readily accomplished (**Fig. 4**), which would facilitate analyzing the full spectrum of gene functions within descendants of a specific lineage with a huge flexibility, whereas the strategy for Cre-lox-regulated conditional RNAi allows gene(s) of interest to be permanently silenced or activated. Moreover, unlike gene knockout, RNAi

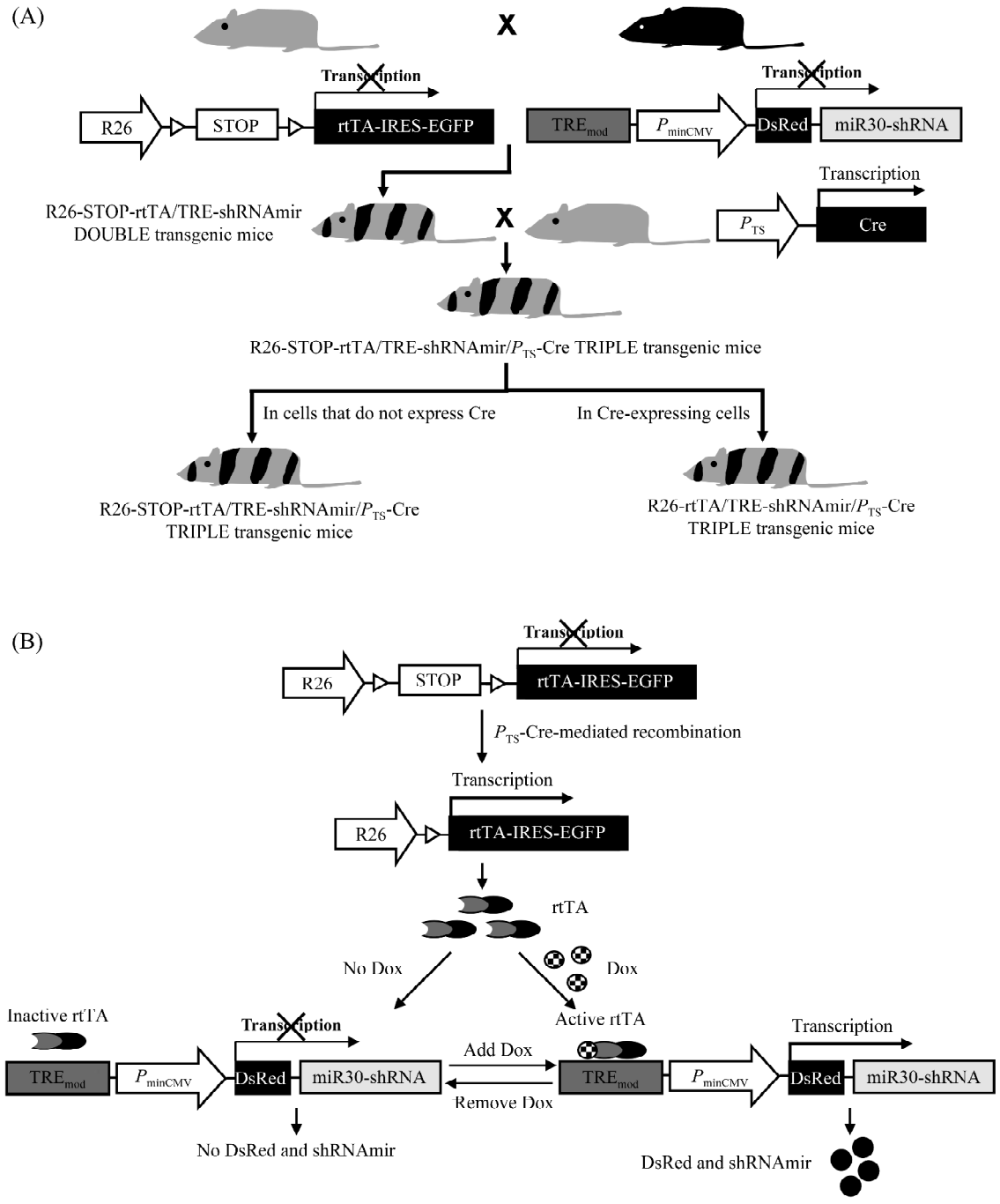

Fig. 4 Strategy for temporal, spatial and lineage-specific RNAi-mediated gene silencing accomplished by combined use of Tet-on system and Cre/*lox* P system

(A) Mating strategy for producing R26-STOP-rtTA/TRE-miR30-shRNA/P_{TS}-Cre TRIPLE transgenic mice. Firstly, the rtTA-IRES-EGFP transgenic mice are bred with homozygous TRE-DeRed-miR30-shRNA transgenic mice to obtain R26-STOP-rtTA/TRE-miR30-shRNA double transgenic mice. Secondly, R26-STOP-rtTA/TRE-miR30-shRNA mice are crossed with any given PTS-Cre transgenic mice to generate R26-STOP-rtTA/TRE-miR30-shRNA/P_{TS}-Cre triple transgenic mice. This mating strategy is very useful to readily realize the loss of gene function in the reversible, temporal, spatial and lineage-specific manner to completely decipher the functions of any given gene(s) in the different cell type or tissue using Cre transgenic mouse strains available. (B) A schematic diagram illustrates the strategy for temporal, spatial and lineage-specific loss of gene function by RNAi. Tissue or cell-type and lineage specificity is provided by Cre-mediated excision of STOP cassette in Cre-expressing cells, and further RNAi against a given gene is temporally regulated by Dox administration. In cells which do not express Cre protein, RNAi is not induced, whereas in Cre-expressing cells, rtTA expression is switched on, and thereafter the active rtTA activates the shRNAmir expression in the presence of Dox, followed by loss of purposed gene function. Other details as in **Figs. 1–3**. ▷, *lox* P site.

strategy does not destroy the gene structure.

Stringent and temporal RNAi-mediated down-regulation of target gene by the improved Tet-based system

Epanchintsev *et al*. [34] generated an all-in-one episomal vector (pEMI, adapted for convenient ligation-free transfer of microRNA cassettes from public libraries) for simultaneously conditional shRNAmir expression and a fluorescent marker protein by modifying the pRTS-1 vector described above [33]. This conditional knockdown-system of the pEMI vector has been confirmed to stringently, reversibly and temporally regulate the expression of miRNAs mediating RNAi, followed by down-regulation of target gene(s), thereby providing a convenient tool to determine gene functions. One major advantage of the system described here is the possibility to realize the conditional knock-down of the gene/protein of interest in one step as all components have been integrated on a single vector. pEMI vector is compatible with recently generated microRNA public libraries (http://www.openbiosystems.com) and will therefore presumably become a widely used tool for conditional RNAi.

Moreover, integrating pEMI vector and Cre/*lox* P system by inserting a *lox* P-flanked transcription STOP cassette sequence (SCS) [18,19] between the bidirectional promoter [P(tet)bi-1] and miR30-shRNA will also permit a temporal, spatial and lineage-specific loss of gene function by shRNAmir-mediated RNAi.

Conclusion and Prospects

The Tet regulatory systems are currently the most widely used regulatory systems for conditional gene expression. Ongoing improvements of the existing components and the continuous addition of new components or other systems, such as Cre/*lox* P system, to expand its range of applicability will make the Tet-based inducible systems more tight, versatile and flexible. Particular applications will include modeling the complex regulatory setups required to analyze sophisticated and multifactorial biological processes in development and disease, consequently not only improving our understanding of living organisms, but also demonstrating some novel and innovative strategies and approaches for the treatment of various human diseases.

References

1 Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 1992, 89: 5547–5551
2 Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science 1995, 268: 1766–1769
3 Albanese C, Hulit J, Sakamaki T, Pestell RG. Recent advances in inducible expression in transgenic mice. Semin Cell Dev Biol 2002, 13: 129–141
4 Bockamp E, Maringer M, Spangenberg C, Fees S, Fraser S, Eshkind L, Oesch F *et al*. Of mice and models: Improved animal models for biomedical research. Physiol Genomics 2002, 11: 115–132
5 Lewandoski M. Conditional control of gene expression in the mouse. Nat Rev Genet 2001, 2: 743–755
6 Ryding AD, Sharp MG, Mullins JJ. Conditional transgenic technologies. J Endocrinol 2001, 171: 1–14
7 van der Weyden L, Adams DJ, Bradley A. Tools for targeted manipulation of the mouse genome. Physiol Genomics 2002, 11: 133–164
8 Baron U, Bujard H. Tet repressor-based system for regulated gene expression in eukaryotic cells: Principles and advances. Methods Enzymol 2000, 327: 401–421
9 Berens C, Hillen W. Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. Eur J Biochem 2003, 270: 3109–3121
10 Xiao D, Sun Y, Gu WW, Chen XG. Tetracycline-controlled transcriptional regulation systems: Countermeasures to eliminate basal transgene leak of Tet-based systems. Progress in Natural Science 2007, 17: 11–19
11 Zhu Z, Zheng T, Lee CG, Homer RJ, Elias JA. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. Semin Cell Dev Biol 2002, 13: 121–128
12 Xiao D, Xu K, Yue Y, Guo ZM, Huang B, Deng XY, Tang H *et al*. Temporal and tight hepatitis C virus gene activation in cultured human hepatoma cells mediated by a cell-permeable Cre recombinase. Acta Biochim Biophys Sin 2004, 36: 687–694
13 Zhu Z, Ma B, Homer RJ, Zheng T, Elias JA. Use of the tetracycline-controlled transcriptional silencer (tTS) to eliminate transgene leak in inducible overexpression transgenic mice. J Biol Chem 2001, 276: 25222–25229
14 Belteki G, Haigh J, Kabacs N, Haigh K, Sison K, Costantini F, Whitsett J *et al*. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. Nucleic Acids Res 2005, 33: e51
15 Mao J, Barrow J, McMahon J, Vaughan J, McMahon AP. An ES cell system for rapid, spatial and temporal analysis of gene function *in vitro* and *in vivo*. Nucleic Acids Res 2005, 33: e155
16 Miyazaki S, Miyazaki T, Tashiro F, Yamato E, Miyazaki J. Development of a single-cassette system for spatiotemporal gene regulation in mice. Biochem Biophys Res Commun 2005, 338: 1083–1088
17 Yu HM, Liu B, Chiu SY, Costantini F, Hsu W. Development of a unique system for spatiotemporal and lineage-specific gene expression in mice. Proc Natl Acad Sci USA 2005, 102: 8615-8620
18 Nagy A. Cre recombinase: The universal reagent for genome tailoring. Genesis 2000, 26: 99–109
19 Sauer B. Inducible gene targeting in mice using the Cre/*lox* system. Methods 1998, 14: 381–392
20 Lee P, Morley G, Huang Q, Fischer A, Seiler S, Horner JW, Factor S *et al*. Conditional lineage ablation to model human diseases. Proc Natl Acad Sci USA 1998, 95: 11371–11376
21 Wakita T, Taya C, Katsume A, Kato J, Yonekawa H, Kanegae Y, Saito I *et al*. Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/*lox*P system. J Biol Chem 1998, 273: 9001–9006

22 Machida K, Tsukiyama-Kohara K, Seike E, Tone S, Shibasaki F, Shimizu M, Takahashi H *et al*. Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins. J Biol Chem 2001, 276: 12140–12146
23 Takaku S, Nakagawa Y, Shimizu M, Norose Y, Maruyama I, Wakita T, Takanot *et al*. Induction of hepatic injury by hepatitis C virus-specific CD8+ murine cytotoxic T lymphocytes in transgenic mice expressing the viral structural genes. Biochem Biophys Res Commun 2003, 301: 330–337
24 Wakita T, Katsume A, Kato J, Taya C, Yonekawa H, Kanegae Y, Saito I *et al*. Possible role of cytotoxic T cells in acute liver injury in hepatitis C virus cDNA transgenic mice mediated by Cre/*lox*P system. J Med Virol 2000, 62: 308–317
25 Chisari FV, Pinkert CA, Milich DR. A transgenic mouse model of the chronic hepatitis B surface antigen carrier state. Science 1985, 230:1157–1160
26 Tan WJ, Chen G, Li GS, Cong Y, Miao J, Du M, Zhan MY. Production of three transgenic mouse lineages which simultaneously carries structural gene and/or nonstructural gene3 (NS3) region of hepatitis C virus. Prog Biochem Biophys 1998, 25: 279–282
27 Tan WJ, Lang ZW, Cong Y, Yi Y, Zhan MY. Transgenic expression of hepatitis C virus core and NS3 proteins in the mouse is not cytopathic. Chinese Journal of Virology 1998, 14: 302–306
28 Dandri M, Lutgehetmann M, Volz T, Petersen J. Small animal model systems for studying hepatitis B virus replication and pathogenesis. Seminars In Liver Disease 2006, 26: 181–191
29 Milich DR. Transgenic technology and the study of hepatitis viruses: A review of what we have learned. Can J Gastroenterol 2000, 14: 781–787
30 Akbar SK, Onji M. Hepatitis B virus (HBV)-transgenic mice as an investigative tool to study immunopathology during HBV infection. Int J Exp Pathol 1998, 79: 279–291
31 Feitelson MA, Larkin JD. New animal models of hepatitis B and C. ILAR J 2001, 42: 127–138
32 Barde I, Zanta-Boussif MA, Paisant S, Leboeuf M, Rameau P, Delenda C, Danos O. Efficient control of gene expression in the hematopoietic system using a single Tet-on inducible lentiviral vector. Mol Ther 2006, 13: 382–390
33 Bornkamm GW, Berens C, Kuklik-Roos C, Bechet JM, Laux G, Bachl J, Korndoerfer M *et al*. Stringent doxycycline-dependent control of gene activities using an episomal one-vector system. Nucleic Acids Res 2005, 33: e137
34 Epanchintsev A, Jung P, Menssen A, Hermeking H. Inducible microRNA expression by an all-in-one episomal vector system. Nucleic Acids Res 2006, 34: e119
35 Knott A, Garke K, Urlinger S, Guthmann J, Muller Y, Thellmann M, Hillen W. Tetracycline-dependent gene regulation: Combinations of transregulators yield a variety of expression windows. Biotechniques. 2002, 32: 796, 798, 800
36 Koponen JK, Kankkonen H, Kannasto J, Wirth T, Hillen W, Bujard H, Yla-Herttuala S. Doxycycline-regulated lentiviral vector system with a novel reverse transactivator rtTA2S-M2 shows a tight control of gene expression *in vitro* and *in vivo*. Gene Ther 2003, 10: 459–466
37 Mizuguchi H, Xu ZL, Sakurai F, Mayumi T, Hayakawa T. Tight positive regulation of transgene expression by a single adenovirus vector containing the rtTA and tTS expression cassettes in separate genome regions. Hum Gene Ther 2003, 14: 1265–1277
38 Salucci V, Scarito A, Aurisicchio L, Lamartina S, Nicolaus G, Giampaoli S, Gonzalez-Paz O *et al*. Tight control of gene expression by a helper-dependent adenovirus vector carrying the rtTA2(s)-M2 tetracycline transactivator and repressor system. Gene Ther 2002, 9: 1415–1421
39 Lena AM, Giannetti P, Sporeno E, Ciliberto G, Savino R. Immune responses against tetracycline-dependent transactivators affect long-term expression of mouse erythropoietin delivered by a helper-dependent adenoviral vector. J Gene Med 2005, 7: 1086–1096
40 Schonig K, Schwenk F, Rajewsky K, Bujard H. Stringent doxycycline dependent control of CRE recombinase *in vivo*. Nucleic Acids Res 2002, 30: e134
41 Wilkinson J 4th, Di X, Schonig K, Buss JL, Kock ND, Cline JM, Saunders TL *et al*. Tissue-specific expression of ferritin H regulates cellular iron homoeostasis *in vivo*. Biochem J 2006, 395: 501–507
42 Kerrison JB, Duh EJ, Yu Y, Otteson DC, Zack DJ. A system for inducible gene expression in retinal ganglion cells. Invest Ophthalmol Vis Sci 2005, 46: 2932–2939
43 Michalon A, Koshibu K, Baumgartel K, Spirig DH, Mansuy IM. Inducible and neuron-specific gene expression in the adult mouse brain with the rtTA2^{S}-M2 system. Genesis 2005, 43: 205–212
44 Mallo M, Kanzler B, Ohnemus S. Reversible gene inactivation in the mouse. Genomics 2003, 81: 356–360
45 Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: Novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci USA 2000, 97: 7963–7968
46 Freundlieb S, Schirra-Muller C, Bujard H. A tetracycline controlled activation/repression system with increased potential for gene transfer into mammalian cells. J Gene Med 1999, 1: 4–12
47 Overbeek PA. Factors affecting transgenic animal production. In: Pinkert CA ed. Transgenic Animal Technology: A Laboratory Handbook. San Diego: Academic Press Inc 1994
48 Schultze N, Burki Y, Lang Y, Certa U, Bluethmann H. Efficient control of gene expression by single step integration of the tetracycline system in transgenic mice. Nat Biotechnol 1996, 14: 499–503
49 Juhila J, Roozendaal R, Lassila M, Verbeek SJ, Holthofer H. Podocyte cell-specific expression of doxycycline inducible Cre recombinase in mice. J Am Soc Nephrol 2006, 17: 648–654
50 Lottmann H, Vanselow J, Hessabi B, Walther R. The Tet-On system in transgenic mice: Inhibition of the mouse pdx-1 gene activity by antisense RNA expression in pancreatic beta-cells. J Mol Med 2001, 79: 321–328
51 Utomo AR, Nikitin AY, Lee WH. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. Nat Biotechnol 1999, 17: 1091–1096
52 Coumoul X, Deng CX. RNAi in mice: A promising approach to decipher gene functions *in vivo*. Biochimie 2006, 88: 637–643
53 Coumoul X, Li W, Wang RH, Deng C. Inducible suppression of Fgfr2 and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre-LoxP system. Nucleic Acids Res 2004, 32: e85
54 Fritsch L, Martinez LA, Sekhri R, Naguibneva I, Gerard M, Vandromme M, Schaeffer L *et al*. Conditional gene knock-down by CRE-dependent short interfering RNAs. EMBO Rep 2004, 5: 178–182
55 Kasim V, Miyagishi M, Taira K. Control of siRNA expression using the Cre-*lox*P recombination system. Nucleic Acids Res 2004, 32: e66
56 Tiscornia G, Tergaonkar V, Galimi F, Verma IM. CRE recombinase-inducible RNA interference mediated by lentiviral vectors. Proc Natl Acad Sci USA 2004, 101: 7347–7351
57 Ventura A, Meissner A, Dillon CP, McManus M, Sharp PA, Van Parijs L, Jaenisch R *et al*. Cre-*lox*-regulated conditional RNA interference from transgenes. Proc Natl Acad Sci USA 2004, 101: 10380–10385
58 Yu J, McMahon AP. Reproducible and inducible knockdown of gene expression in mice. Genesis 2006, 44: 252–261
59 Coumoul X, Shukla V, Li C, Wang RH, Deng CX. Conditional knockdown of Fgfr2 in mice using Cre-LoxP induced RNA interference. Nucleic Acids

Res 2005, 33: e102
60 De Souza AT, Dai X, Spencer AG, Reppen T, Menzie A, Roesch PL, He Y *et al*. Transcriptional and phenotypic comparisons of Ppara knockout and siRNA knockdown mice. Nucleic Acids Res 2006, 34: 4486–4494
61 Xia XG, Zhou H, Samper E, Melov S, Xu Z. Pol II-expressed shRNA knocks down Sod2 gene expression and causes phenotypes of the gene knockout in mice. PLoS Genet 2006, 2: e10
62 Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D *et al*. Second-generation shRNA libraries covering the mouse and human genomes. Nat Genet 2005, 37: 1281–1288
63 Li MZ, Elledge SJ. MAGIC, an *in vivo* genetic method for the rapid construction of recombinant DNA molecules. Nat Genet 2005, 37: 311–319
64 Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. Proc Natl Acad Sci USA 2005, 102: 13212–13217

Edited by
Minghua XU