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FAST (Flexible Accelerated STOP TetO-knockin): a versatile and efficient new gene modulating system

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Abstract

We created the FAST (Flexible Accelerated STOP TetO-knockin) system, an efficient method for manipulating gene expression *in vivo* to rapidly screen animal models of disease. A single gene targeting event yields 2 distinct knockin mice –STOP-tetO and tetO knockin– which permit generation of multiple strains with variable expression patterns: 1) knockout, 2) Cre-mediated rescue; 3) tTA-mediated misexpression; 4) tTA-mediated overexpression; and 5) tTS-mediated conditional knockout/knockdown. Using the FAST system, multiple gain- and loss-of-function strains can therefore be generated on a timescale not previously achievable. These strains can then be screened for clinically-relevant abnormalities. We demonstrate the flexibility and broad applicability of the FAST system by targeting several genes encoding proteins implicated in neuropsychiatric disorders: Mlc1, Neurologin 3, the serotonin 1A receptor, and the serotonin 1B receptor.

Keywords

genetics; gene targeting; animal model; mouse; conditional modulation of gene expression; developmental change

Gain-of-function and loss-of-function studies are commonly used to examine gene function *in vivo*, particularly in attempts to model human disease in animals. Developing animal models of disease is key to the process of elucidating neuropsychiatric disease pathophysiology, in turn leading to drug discovery and translation to patient populations.

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Supplementary information: Methods and discussions about the specificity and limitations of the FAST system

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However, these studies typically involve generating separate lines of transgenic mice that over- or under-express the gene of interest, a process that can take several years. Increasing the speed of this screening process is of utmost importance for development of new neuropsychiatric medications based on novel genetic targets. Using a new technological approach, we circumvented this problem by generating several different mouse strains from one single gene-targeting event. This system– FAST (Flexible Accelerated STOP TetO-knockin)– includes the single gene targeting event and five versatile applications (Fig 1A). It allows us to take advantage of established Cre-recombinase, tTA (tetracycline-controlled transcriptional-activator), and tTS (tetracycline-controlled transcriptional-silencer) lines to rapidly produce 5 separate lines of mice from the original knock-in: 1) knockout; 2) Cre-mediated rescue; 3) tTA-mediated ectopic expression; 4) tTA-mediated overexpression; and 5) tTS-mediated conditional knockout/knockdown.

The FAST system allows us therefore to rapidly generate multiple lines of mice that provide a spectrum of expression levels for single genes, from selective knockout to selective overexpression. In addition, the FAST system has the added advantage of easily integrating temporal and spatial specificity into the manipulations of gene expression. In this paper, we demonstrate the efficacy of the FAST system using multiple genes implicated in neuropsychiatric disorders. One of our overall goals is to use the FAST system to make mouse models using genes that have been linked to disease, but 1) have unknown function and 2) have not yet been knocked out in mice. We therefore chose to start with the *Mlc1* gene as our initial test of the system, since its function is unknown (though homology to other proteins suggests that it may be an integral membrane transporter (1)), and knockout mice have not yet been made. Mutations in this gene have been associated with megalencephalic leukoencephalopathy with subcortical cysts, an autosomal recessive neurological disorder (1); *Mlc1* has also been implicated in catatonic schizophrenia (2). We describe *Mlc1* gene targeting in detail as proof of principle of efficacy of the FAST system. Moreover, we demonstrate the broad applicability of the FAST system by targeting three additional genes of importance in neuropsychiatric disorders: the serotonin 1A receptor (depression, anxiety); the serotonin 1B receptor (addiction, impulsive aggression, OCD); and Neurologin 3 (autism) (3).

One of our goals was to obtain two distinct types of regulatable knock-in mice from a single knock-in gene targeting event. To achieve the single gene targeting event, a loxP-FRT-Neo STOP-FRT-tetO-loxP cassette (Figure S1 in Supplement 1) was inserted immediately upstream of the translation initiation site in exon 2 of the *Mlc1* gene using conventional homologous recombination, resulting in *Mlc1* STOP-tetO knock-in mice (Fig 1B; see also Figure S2 in Supplement 1). By crossing *Mlc1* STOP-tetO knock-in mice with Flippase expressing mice (ROSA-Flpe mice) (4), we removed the FRT-flanked Neo STOP minigene through Flippase-FRT recombination. This cross yielded *Mlc1* tetO knock-in mice (Fig 1C). These results demonstrated that one gene targeting event yielded two distinct mouse lines: STOP-tetO and tetO knock-in.

To validate application #1(knockout), we examined *Mlc1* expression levels at postnatal day 28. In wild type mice, *Mlc1* mRNA was expressed only by astrocyte-lineage cells: astrocytes, Bergmann glia and ependymal cells (Fig 2A; see also Figure S4 in Supplement 1) as detected by *in situ* hybridization. In *Mlc1* STOP-tetO homozygous mice, *Mlc1* mRNA was not detected at any age examined, since the STOP sequence terminates transcription (5) driven by the endogenous *Mlc1* promoter (Fig 2A; see also Figures S3 and S6 in Supplement 1). This indicates that STOP-tetO knock-in mice act like straight null mice, as reported in previous studies using STOP sequence insertion (application #1) (6,7).

STOP-tetO knock-in mice permit us to conduct 2 further manipulations: Cre-mediated rescue (application #2, Fig 2B) and tTA-mediated ectopic expression (application #3, Fig 2C). To test Cre-mediated rescue, we crossed Mlc1 STOP-tetO mice with two established Cre lines: Emx1-Cre (8) (expresses Cre mainly in forebrain) and HSP70-Cre (9) (expresses Cre in germline). We then examined Mlc1 mRNA levels in the resulting homozygous STOP-tetO::Cre mice. Emx1-Cre-mediated recombination resulted in a partial rescue as illustrated by expression in the Bergmann glia (Fig 2B); germline Cre-mediated recombination resulted in complete rescue (Fig. 2B). These results indicate that crossing with different Cre lines leads to recovery of endogenous gene expression corresponding to the particular Cre expression pattern (Figure S4 in Supplement 1) (6,7).

To test application #3 (tTA-mediated ectopic expression), we used α CamKII-tTA mice (10), which express tTA in forebrain neurons. Fig 2C demonstrates that tTA expression driven by the α CamKII neuronal promoter leads to ectopic Mlc1 mRNA induction in neuronal cells—e.g., hippocampal CA1 neurons (arrow) and striatum medium spiny neurons (arrowhead). Thus, by using a strain in which tTA is expressed in the same region or cell type as native Mlc1 (e.g. glia), we can conduct tTA-mediated rescue experiments (11). By using mice in which tTA is expressed in a different region or cell type than the targeted gene (e.g. neurons), we can conduct tTA-mediated ectopic expression studies.

To perform application #4 (tTA-mediated overexpression) and application #5 (tTS-mediated conditional knockdown), one condition was required: insertion of the tetO sequence upstream of the translation initiation site does not alter wild type expression patterns. Since insertion of tetO, which consists of a tetracycline operator site and CMV minimal promoter (12), may change transcription efficiency, we compared gene expression levels with or without tetO sequence insertion *in vivo*. Using four different tetO knock-in mice, we have demonstrated the robustness of this system: 1) tetO insertion does not affect overall protein expression levels, which was not necessarily expected, and 2) the change in 5'-UTR due to tetO insertion does not affect mRNA splicing, as demonstrated both by Western blot and DNA sequencing; in addition, the insertion does not affect the mRNA expression pattern (in Supplement 1, see supplementary text and Figures S4, S6, S7, and S8).

To test application #4, tTA-mediated overexpression, we generated astrocyte specific tTA mice (Mlc1 mtTA BAC transgenic mice, see Supplementary Methods in Supplement 1), crossed them with Mlc1 tetO knock-in mice, and examined levels of Mlc1 mRNA. Fig 2D and 2E show the expression of Mlc1 mRNA in Mlc1-mtTA::Mlc1-tetO mice in the presence of doxycycline (DOX) and absence of DOX, respectively. In the presence of DOX, tTA cannot bind the tetO sequence, preventing additional trans-activation of Mlc1 (Fig 2D). In the absence of DOX, tTA binds tetO and initiates transcription in excess of endogenous transcription, resulting in Mlc1 overexpression in astrocytes (Fig 2E; see also Figure S5 in Supplement 1).

To validate application #5, conditional knockout/knockdown, we used a tTS system. tTS is a tetracycline-dependent transcriptional-silencer containing the Kruppel-associated box (KRAB) domain of human zinc finger protein 10 (13). KRAB works as a transcriptional silencer extending over regions of a radius of 3 kilobases when tethered to DNA. The binding of tTS protein to DNA is also reversibly controlled by DOX (Fig. 2F and 2G). In Actin-tTS (14)::Mlc1-tetO homozygous mice, tTS protein was expressed ubiquitously under the control of human β -actin promoter. In the absence of DOX, tTS binds tetO sites and the Mlc1 promoter is widely suppressed; mRNA and protein were not detectable by *in situ* hybridization (Fig 2G), RT-PCR (Figure S6 in Supplement 1), or Western blotting (Figure S7 in Supplement 1). Thus Actin-tTS::Mlc1-tetO mice without DOX function as knockout mice. In the presence of DOX, tTS cannot bind tetO sites and the Mlc1 promoter drives

transcription, resulting in wild type Mlc1 expression (Fig 2F). In the case of the serotonin 1A receptor, we have shown that the tTS system can be used to produce inducible and reversible tissue-specific knockouts/knockdowns by using mice that express tTS selectively in the raphe nuclei or in the hippocampus (Richardson-Jones *et al*, in press (15)).

The FAST system described here demonstrates versatile manipulation of gene expression. A single gene targeting event yields two distinct knock-in mice, ultimately resulting in at least 5 different types of gene manipulations. Applications requiring tTA or Cre mice can take advantage of diverse mouse lines already available as common sources (<http://www.zmg.uni-mainz.de/tetmouse/tet.htm>, <http://nagy.mshri.on.ca/cre/>). This versatility will serve as a template for a systematic approach to performing *in vivo* gain and loss of function studies.

These five different applications allow us to perform a variety of different studies of gene expression. For example, unlike the Cre-loxP system, which causes an irreversible knockout, the tTS system allows reversible gene knockout; this is useful not only in developmental studies, but also in temporary gene knockout/knockdown and recovery experiments, mimicking disease conditions and recovery with or without treatments.. An additional advantage of the tTS system over knockout strategies employing inducible recombinases (such as Cre-ER) is that the level of knockout achieved seems to be superior; this is based on the fact that we see between 90–100% suppression using our tTS system (Richardson-Jones *et al* in press (15); and unpublished data), versus at most 50% recombination efficiency with the CreER-loxP system.

In summary, we believe that the FAST system will accelerate our ability to dissect the circuits and mechanisms underlying various neuropsychiatric diseases, and ultimately lead to the production of new treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. FAST system

(A) Diagram of diverse applications of FAST system

A single gene targeting event yields two distinct knock-in mice (STOP-tetO and tetO), which ultimately yield 5 different applications.

(B) Strategy for insertion of STOP-tetO cassette into *Mlc1* locus (*Mlc1* STOP-tetO knock-in mouse)

Open triangles represent loxP sites. Filled triangles represent FRT sites. Neo is the PGK-EM7-NEO minigene and is in the sense orientation. STOP is the cassette containing elements designed to terminate both transcription and translation. ATG represents the translation initiation site. tetO is the cassette containing the tetracycline operator site and CMV minimal promoter. The STOP-tetO cassette (3.5 kb) was inserted just upstream to ATG.

(C) *Mlc1* tetO knock in mouse

The Neo-STOP minigene was removed by crossing with ROSA-Flpe mice, yielding flippase-FRT recombination. The tetO sequence (600 bp) remained upstream to ATG.

Fig. 2. Five distinct gene manipulation strategies are created from a single knock-in event

(A) STOP-tetO knock in mice are indistinguishable from straight knockout mice. The STOP cassette terminates transcription. Images show Mlc1 ISH in the cerebellar lobe of STOP-tetO homozygote and wild type mice. Note that Mlc1 mRNA is expressed in astrocyte lineage cells. Scale: 200 μ m.

(B) Cre-mediated rescue.

Cre protein excises the loxP flanking STOP-tetO cassette, and transcription is rescued. Mlc1 ISH of Emx1-Cre::Mlc1 STOP-tetO and HSP70-Cre::Mlc1 STOP-tetO homozygotes is shown.

(C) tTA-mediated ectopic expression

DOX regulates tTA protein binding to the tetO sequence and subsequent transactivation (DOX-, DOX+). Ectopic neuronal Mlc1 mRNA induction was observed in α CamKII-tTA::Mlc1 STOP-tetO homozygote. Scale: 1 mm.

(D, F) tetO knock in mice are indistinguishable from wild type mice. In the presence of doxycycline, tTA and tTS cannot bind the tetO site, and the level of Mlc1 expression is the same as that seen in wild type mice. Images demonstrate Mlc1 ISH in cerebral cortex and cerebellum. Scales: 1 mm in cerebral cortex; 200 μ m in cerebellum.

(E) tTA-mediated overexpression

In the absence of DOX in Mlc1-mtTA::Mlc1 tetO homozygotes, tTA transactivates Mlc1 mRNA in excess of transcription driven by the endogenous promoter.

(G) tTS-mediated knockout

In the absence of DOX, tTS suppresses transcription in Actin-tTS::Mlc1 tetO homozygotes.