Mapping Transgene Insertion Sites Reveals the α -Cre Transgene Expression in Both Developing Retina and Olfactory Neurons

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Article

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Abstract

The Tg(Pax6-cre,GFP)2Pgr (α -Cre) mouse (MGI:3052661) is a commonly used Cre line thought to be retinal-specific. Using targeted locus amplification (TLA), we mapped the insertion site of the transgene, and defined primers useful to deduce zygosity. Further analyses revealed four tandem copies of the transgene. The insertion site mapped to clusters of vomeronasal and olfactory receptor genes. Using R26R and Ai14 Cre reporter mice, we confirmed retinal Cre activity, but also detected expression in olfactory neurons, implicating the influence of neighbouring regulatory elements. RT-PCR and the buried food pellet (BFP) test did not detect any effects of the transgene on flanking genes in the nasal mucosa and retina. Together, these data precisely map α -Cre, show that it does not affect surrounding loci, but reveal previously unanticipated transgene expression in olfactory neurons. The α -Cre mouse can be a valuable tool in both retinal and olfactory research.

Introduction

Cre/LoxP technology is widely used in the field of mouse genetics. Both spatial and temporal genetic manipulations can be performed using cell-type-specific enhancers/promoters to drive Cre recombinase expression in combination with Cre recognition (loxP) sites in interest genes¹. Many retinal cell-targeted Cre transgenic mice have been created to facilitate the study of retinal development and retinal diseases². The Tg(Pax6-cre,GFP)^{2Pgr} (α -Cre) mouse (MGI:3052661) is a commonly used retina-specific Cre line³. In addition to the α enhancer, the α -Cre transgene construct also has the P0 promoter of the Pax6 gene, an IRES-GFP cassette, an intron from the Hbb (β -hemoglobin) gene, and an SV40 poly (A) sequence³. The α -Cre transgene is active from embryonic day 10 (E10) in peripheral retinal progenitors that give rise to all cells in the mature retina of this region^{3,4}. In the mature eye, the α -Cre is also active in cells of the ciliary body^{3,4} and GABAergic amacrine cells⁵.

The α -Cre mouse line is generated via pronuclear microinjection of the α -Cre transgene construct³. Therefore, the integration site of the α -Cre transgene is random and unknown. Such insight is helpful to deduce whether the transgene might have deleterious effects, and to define primer pairs around the integration site to determine whether breeders are heterozygous or homozygous for the transgene.

Previously, we reported that Rb gene knockout (KO) in the retina with α -Cre (α -Cre; Rb^{f/f}) induces ectopic division and apoptosis of many retinal excitatory neurons⁶. In order to elucidate the mechanism of RbKO-induced retinal cell death, we bred α -Cre;Rb^{f/f} mice with Bax^{-/-} mice⁷. Bax is a member of Bcl2 family proteins and mediates neuronal cell death⁸, including physiological retinal apoptosis^{9,10} and neuronal death in the Rb/p107 double knockout brain¹¹. Bax-null males are infertile¹². To generate α -Cre; Rb^{f/f};Bax^{-/-} mice, we first generated α -Cre;Rb^{f/f};Bax^{+/-} males and Rb^{f/f};Bax^{-/-} females, and inter-bred them. Of 96 pups, we obtained 50 pups with α -Cre and 48 pups with Bax^{-/-} alleles as predicted, but only five pups (5.2%) with α -Cre;Bax^{-/-}, far less than the expected 24 pups (25%), suggesting a genetic linkage between the α -Cre transgene and the Bax locus on chromosome 7⁷. This result prompted us to

investigate the precise location of the *α-Cre* transgenic insertion site and any possible functional consequences of the genetic recombination.

Commonly used methods for identifying integration sites include inverse PCR^{13–15}, whole-genome sequencing and capture-based targeted re-sequencing^{16,17}. However, these methods are cumbersome and largely unsuccessful¹⁸. Recently, several studies have employed targeted locus amplification (TLA) to map integration sites of different Cre or other transgenes^{18–20}. TLA selectively amplifies and sequences entire genes based on the crosslinking of physically proximal sequences and works without detailed prior locus information²¹. TLA can generate complex DNA libraries covering >100kb of contiguous sequence surrounding one primer pair complementary to a short locus-specific sequence²². In TLA, crosslinked genomic DNA is digested into ~0.2 kb small fragments by the restriction enzyme *NlaIII* and ligated to form larger circular DNA. These DNA fragments are de-crosslinked and digested by *NspI* to create ~2 kb fragments, which are then subjected to PCR with primers derived from sequences unique to the transgene. The PCR product is sequenced and mapped to the genome, thereby localizing the transgene and also revealing insertions, deletions or other structural rearrangements both within the transgene and in flanking sequences^{21,22}.

In this study, we identified the genomic insertion site of the *α-Cre* transgene on mouse chromosome 7 by TLA analysis. We found it is among clusters of vomeronasal receptor 2 (*Vmn2r*) genes and olfactory receptor (*Olfir*) genes. Indeed, the *α-Cre* transgene is also expressed in many olfactory neurons. Notably, the *α-Cre* transgene has no significant effects on the expression of flanking genes and olfactory function.

Results

Mapping the integration site of the *α-Cre* transgene by TLA

To identify the precise *α-Cre* integration site, we performed TLA analysis on spleen cells from P8 *α-Cre* mice. Two primer sets complementary to the Cre gene sequences were designed (Figure 1A). Primer set 1 included primer RV461 and FW907; primer set 2 included primers RV330 and FW408. Both were used to generate products that were sequenced to a depth of 2Mb (Figure 1B). We mapped the sequence data to the mouse genome (Version mm10) to analyze the integration site and to confirm the component sequences of the *α-Cre* transgene. TLA revealed that the *α-Cre* transgene is inserted inversely into mouse chromosome 7 (Chr7: 85259496-85287099) and that the insertion is accompanied by a 27kb deletion (Figure 1C). There are no annotated genes in the integration region. No single nucleotide variant was seen in the Cre sequence. From the current dataset TG (transgene)-TG fusions could not be accessed.

To confirm the insertion, we designed primers flanking the predicted left and right junctions (Table 1). Primer 1 targets the left junction but combining it with several primers from the SV40 polyA region (Table 2) did not generate any PCR products (Figure 2A and data not shown). Primer 1 and primer 5 from the *Hbb* gene intron generated a 722bp PCR product which was absent in wild type animals (Figure 2B). Sequencing of this 722bp PCR product revealed that chromosome 7 sequence is linked through an 18bp

linker (AGGCTGATTATGATCATC) to the *Hbb* intron (Figure 2C), and not, as anticipated, through the SV40 poly A sequence (Figure 1A). Primer 1 and 2 (from the deleted region) generated a 250bp PCR product in wild type animals, but not α -Cre homozygotes (Figure 2B). Together these data reveal the precise left junction of the α -Cre transgene in chromosome 7, and provide a primer set that is useful to define zygosity.

Table 1
Primers for differentiating zygosity of α -Cre transgene

Primer	Location	Junction (Figure 2A)	Sequence
Primer 1	Chromosome 7	right junction	TGAATCCTGCCTATCATGTG
Primer 2	Chromosome 7	right junction	CCCCAAAATTCCTCAAAGA
Primer 3	Chromosome 7	Left Junction	ATTGAGATGGGCTGCTCTGT
Primer 4	Chromosome 7	Left Junction	GCTCCATCTTCACCCCTGTA
Primer 5	α -Cre transgene	right junction	CACCATGGACCCCATGATAA
Primer 6	α -Cre transgene	Left Junction	AATTGCATCGTCACGACAAA

Table 2
Primers to map the entire transgene sequence

Amplified for	No.	Primer	Primer sequence(5'→3')
Breakpoint at α -enhancer	1	F40	GATGCCCATCAGGAGACAGT
	2	F154	GCTCCATCTTCACCCCTGTA
	3	WT R382	ATTGAGATGGGCTGCTCTGT
	4	WT R471	AAACGTTTTGAAGGGGGAGT
	5	R308	AGGGCAAGGCCTACAGAAAG
	6	R351	GCAAGAGTGGACATTGCACA
	7	R379	ATCTGGGCTCTGGCTGTCT
	8	R473	AATTGCATCGTCACGACAAA
	9	R841	TTTCCACTTCCTCGGACATC
	10	R1164	TTGGAAATCCCACTCACACA
Breakpoint at <i>Hbb</i> intron	1	R540	TGAATCCTGCCTATCATGTG
	2	WT F5	GGGAAATCAGGGATACAGAGG
	3	WT F257	CCCCAAAATTCCTCAAAAGA
	4	U1R	TCTCTTTCCTACAGCTCCTTGG
	5	F66	GGGCAAAGTTTTTCAGGGTGT
	6	F115	CACCATGGACCCCATGATAA
	7	F36	GTGCTGGTTGTTGTGCTGTC
	8	F175	TCAGCCTTCCAGAACTGTTG
SV40 polyA	1	SV40-1F	ACCTGAAACATAAAATGAATGCAA
	2	SV40-2F	ACCGTCGACCTCGAGAAGTA
	3	SV40-R	CCAGACATGATAAGATACATTGATGA
Linker-1	1	BRAZ11-F	GGATATTAAGGAAAGTTAGCGCC
	2	BRAZ20-F	TCGTTGACATTTAACTCTGGGGCAGGTCCTCGCG
	3	BRAZ12-R	CCGGTTATTCAACTTGCACCATGCCGCCC
	4	BRAZ19-R	GAACCTCATCACTCGTTGCATCGACCGG
Linker-2	1	IRES-R134	GGGAAAGACCCTAGGAATGC
	2	IRES-R188	GAGGAACTGCTTCCTTCACG

Amplified for	No.	Primer	Primer sequence(5'→3')
	3	Cre-F228	GCGCTAAGGATGACTCTGGT
	4	Cre-F323	ATACCGGAGATCATGCAAGC
Linker-3	1	GFP-R160	ATGGCGCTCTTGAAGAAGTC
	2	GFP-R278	TTCCCTTAAGCTCGATCCTG
	3	IRES-F393	AATGGCTCTCCTCAAGCGTA
	4	IRES-F401	TAACAAGGGGCTGAAGGATG
Linker-4	1	GFP-F518	CCCAACGAAAAGAGAGACCA
	2	GFP-F285	TCAAGGAGGACGGAAACATC
	3	BG-R071	AATCAAGGGTCCCCAACTC
	4	BG-R122	CCATGGTGATACAAGGGACA

To map the right junction, we used primer 4 from chromosome 7, and primer 6 from the α -enhancer (Figure 2A), which generated a 436bp PCR product in heterozygotes and homozygotes of the α -Cre transgene, but no product from wild type DNA (Figure 2D). However, using primer 4 with primer 3 from the deleted region (Figure 2A) generated a 250bp PCR product both in wild type, heterozygotes and homozygotes of α -Cre transgenes (Figure 2D). This was unexpected, but Blast analysis using the UCSC genomic browser found that the 500bp sequence around the right junction has multiple copies along chromosome 7 (Figure 2E). These repetitive sequences are in introns or non-coding regions around *Vmn2r27-68* genes. These data confirm the chromosome 7 location of the right junction, but while Primers 4+6 are useful to confirm transgene presence, the repetitive nature of the flanking sequence precludes the use of primers in this area (e.g. 3+4) to define zygosity. The left junction solves this issue: Primers 1+5 can confirm transgene presence, and Primers 1+2 can determine zygosity, as this amplicon is present in heterozygotes, but absent in homozygotes (Figure 2A, B).

Determining the SV40 polyA site and copy number of the α -Cre transgene

Using multiple primer sets to amplify overlapping fragments (Table 2), we sequenced the α -Cre transgene from the α enhancer to the *Hbb* intron, without the SV40 polyA signal. The entire sequence of this stretch was 4911 bp, including 5 linkers (Figure 3A). The lack of the SV40 polyA signal at the left junction with chromosome 7 suggests it was deleted during integration (Figure 2C). TLA detected SV40 polyA sequence, but both flanking regions were vector sequences without any chromosome 7 elements, suggesting tandem integration.

To confirm the presence of SV40 polyA sequence and to test for tandem arrays, we designed primers in the *Hbb* intron, SV40 polyA tail and the α -enhancer (Figure 3B). Combining forward primers in the *Hbb* intron (F66 and F36) with a reverse primer in the SV40 poly A tail (SV40-R) robustly amplified the

expected fragments (Figure 3C). In contrast, combining forward *Hbb* primers with forward SV40 primers (SV40-1F, SV40-2F) gave only weak non-specific bands (Figure 3C). Similarly, a reverse primer in the α -enhancer generated specific PCR products with SV40-1F/2F primers, and not with SV40-R primer (Figure 3D), supporting the existence of tandem transgene copies. Interestingly, several PCR products were obtained with R351/SV40-1F, but only one with R351/SV40-2F (Figure 3D), suggesting there were several forms of the SV40- α enhancer junction. This was confirmed with primers F36/R351 designed to amplify the entire α -enhancer-SV40-*Hbb* region, and which detected long, medium and short PCR products (Figure 3E). DNA sequencing these three amplicons revealed that, relative to the long PCR product, the medium version lacked 48bp, which included part of vector sequence 2 (V2) adjacent to the SV40 polyA signal and part of the α enhancer, while the short version lacked 281bp, which included part of the SV40 polyA signal, all of V2, and part of the α -enhancer (Figure 3F). These differences at the junctions of tandem copies created variable α -enhancer sizes of 1647 bp, 1621 bp and 1463 bp (Figure 3F). The α enhancer sequence linked to chromosome 7 (Figure 2A and 3A) is similar to the short version, and is 1465 bp. These data indicate at least 4 copies of the α -Cre transgene, but only 3 copies of the SV40 polyA signal. Indeed, quantitative real-time PCR analysis of a 143bp fragment from the Pax6 P0 promoter showed that the average transgene copy number is about 4.5 (4.52 ± 1.29) for heterozygotes ($n=38$), and 8.2 (8.205 ± 1.05) for homozygotes ($n=26$) respectively (Mean $\pm$ SD) (Figure 3G). Multiple copies of transgenes are frequently integrated in a head-to-tail tandem array at a single genomic site²³. In summary, our data indicate that there are 4 tandem copies of the α -Cre transgene, although further work would be required to deduce the exact order of the three versions of the SV40- α enhancer junction (Figure 3H).

Expression of the α -Cre transgene in the retina and the olfactory system

The α -Cre transgene integration site (Figure 2) is among *Vmn2r* and olfactory receptor (*Olfir*) gene clusters (Figure 3I). 4C (circular chromosome conformation capture) technology was first developed to study gene interactions between the *Hbb* gene on qE3 and other genes on chromosome 7²⁴. They found that multiple *Olfir* genes on chromosome 7 interact with the *Hbb* gene, including those flanking the α -Cre transgene (Figure 3I). Therefore, it is possible that regulatory elements of these olfactory receptor genes may induce α -Cre transgene expression in olfactory neurons. To address this issue, we crossed the α -Cre line with two commonly used Cre reporter lines, *Gt(ROSA)26Sor^{tm1Sor}*, also referred to as *R26R*²⁵ and B6.Cg-*Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze/J}* mouse, also referred to as *Ai14*²⁶, to obtain α -Cre;*R26R* and α -Cre;*Ai14* double transgenic mice. We then examined α -Cre expression pattern at different time points, first in the retina to confirm the expected patterns with these reporters, and then the olfactory system to search for ectopic transgene activation.

At embryonic day 12 (E12), horizontal sections through α -Cre;*Ai14* mouse heads confirmed, as expected, that α -Cre is active in nasal and temporal peripheral retina (Figure 4A). Retinal ganglion cells (RGCs) and their axons (optic nerve and optic chiasm) were also tdTomato-positive. There was no expression in lens, cornea or any other tissue in the eye. X-gal staining of horizontal sections of α -Cre;*R26R* eyeballs revealed a similar pattern at E14 (Figure 4B). At the day of birth (post-natal day 0, P0), nasal and temporal peripheral retina of α -Cre;*R26R* mice exhibited deep blue staining, while the dorsal and ventral retina had

no staining (Figure 4C). This was consistent with results from wholemount analyses of *α-Cre;Ai14* retina at P18 (Figure 4D). By comparing the expression of GFP and tdTomato in *α-Cre;Ai14* retinas at E12, we found the area of GFP expression was significantly smaller than that of tdTomato (Figure 4E). GFP represents current expression level of the *α-Cre* transgene, while tdTomato marks lineage. Down-regulation of retinal *α-Cre* expression is well known³, and we also confirmed that silencing has begun even by E12, especially in retinal progenitor cells (RPCs) close to the central retina. GFP exhibited a distal^{high}-proximal^{low} expression gradient, in accordance with the *α*-enhancer-dependent *Pax6* expression gradient across the optic cup⁴.

At E16, bright GFP was also evident at the inner side of the NBL, including the central retina (Figure 4F). Co-staining with the amacrine cell marker, AP2 α , showed that GFP was expressed in amacrine cells from E16 (Figure 4F, 4G). These cells were Ki67 negative, suggesting they had exited the cell cycle (Figure 4H). Spatially, GFP⁺ amacrine cells were found across the whole retina without any apparent gradient (Figure 4F), suggesting it was controlled by the *Pax6* P0 promoter, and as reported previously^{3,4}. These data show that the *α-Cre* mice in our group behave as expected with respect to retinal expression.

The murine olfactory system has two distinct chemosensory systems, referred to as the main and accessory olfactory systems. The former consists of main olfactory sensory epithelium (MOE) and the septal organ of Masera (SOM), all projecting to the olfactory bulb (OB). The accessory olfactory system consists of the vomeronasal organ (VNO) and the ganglion of Grüneberg (GG), all projecting to accessory olfactory bulb (AOB)^{27,28}. Olfactory sensory neurons (OSNs) located in these four areas of the epithelium lining the nasal cavities (MOE, VNO, SOM and GG), express different repertoires of chemosensory receptors which can recognize thousands of odorant molecules or pheromones that vary in shape, size, charge and function^{27,28}. The major chemosensory receptors include odorant or olfactory receptors (Olfr) mainly expressed in MOE, and vomeronasal receptors (Vmnr) mainly expressed in VNO²⁸. The vast majority of MOE neurons express Olfr, an enormous family of rhodopsin-like G protein-coupled receptors (GPCRs)²⁹. Rodents have >1,000 *Olfr* genes dispersed on almost every chromosome in clusters varying in size from one gene to hundreds of genes²⁹. VNO sensory neurons detect stimuli mainly by using two families of GPCRs including Vmnr type 1 and 2 (Vmn1Rs and Vmn2Rs). Vmn1Rs are a large family of ~180 GPCRs in mouse and are distantly related to bitter taste receptors. Vmn2Rs are a similarly large family of ~120 GPCRs in mouse, are distantly related to the sweet taste receptor. Each Vmn1R is expressed in a small subset of sensory neurons confined to the apical VNO zone²⁹, while most Vmn2Rs are expressed in dispersed subsets of basal VNO sensory neurons³⁰.

As the closest genes to the *α-Cre* transgene were *Vmn2r68* and *Vmn2r69*, and the *α-Cre* transgene was inserted in the *Vmn2r65-73* gene cluster (Figure 3), we first assessed the VNO of *α-Cre;Ai14* mice. At E16, the *α-Cre* transgene was active in both the retina and the VNO (Figure 5A-B). The intensity of tdTomato in the VNO was lower than retina, suggesting weaker ROSA locus transcriptional activity in the former (Figure 5A-B). Similar to the retina (Figure 4E), there were less GFP⁺ than tdTomato⁺ cells in the VNO, suggesting that the *α-Cre* transgene is also gradually silenced in this context (Figure 5B-D). Then we

examined the main olfactory sensory epithelium (MOE) as the α -Cre transgene was located in the *Olf291-290* and *Olf300-292* clusters (Figure 3). Indeed, transgene activity was evident in some cells of the nasal olfactory epithelium (Figure 5E-H).

More tdTomato⁺ cells were observed in the VNO than MOE (Figure 5B, F), perhaps reflecting the difference in proximity of α -Cre to the *Vmn2r* and *Olf* gene clusters on chromosome 7 (Figure 3B). Like the optic nerve (Figure 4A, 5A, 5E, 5F, 5H), OSN axons were tdTomato⁺, including the olfactory nerve projecting to olfactory bulb (Figure 5I-J), as well as in vomeronasal nerve projecting to accessory olfactory bulb (Figure 5K-L). However, we did not detect tdTomato in the SOM or GG (data not shown). We also detected GFP in OSN axons (Figure 5J, 5L) indicating that α -Cre is expressed in these mature neurons, akin to retinal amacrine cells, but in contrast to retinal ganglion cells (Fig. 5G). Overall, these data reveal that α -Cre is active in a subset of olfactory cells and, like the retina, is also expressed in some mature neurons.

The α -Cre transgene has no significant effect on neighboring endogenous genes

Expression of α -Cre in olfactory neurons reflects the regulatory elements of neighboring endogenous gene clusters on the transgene. Thus, we next assessed whether the corollary applies: does the α -Cre transgene affect the expression of olfactory genes? Using quantitative RT-PCR, we found that mRNA levels of 4 flanking *Vmn2r* genes (*Vmn2R67*, *68*, *69*, *70*), 2 flanking *Olf* genes (*Olf291*, *Olf300*) and the *OMP* (olfactory marker protein) were not different between α -Cre⁺ and α -Cre⁻ mice in the retina and nasal mucosa (Figure 6A). This result suggested that the α -Cre transgene has no significant effects on adjacent genes, although these data do not exclude more distal effects.

There are many chemosensory receptor genes on chromosome 7 (Figure 3). Thus, to further explore the possibility of transgene interference, we employed the buried food pellet (BFP) test^{31,32} to examine olfactory function. Based on the average mobility scores (quadrants/s), there was no significant difference between α -Cre⁺ and α -Cre⁻ mice ($p > 0.05$) (Figure 6B). The average latency time to find buried pellets across three trials also showed no significant difference between α -Cre⁺ and α -Cre⁻ mice ($p > 0.05$) (Figure 6C). Together, our molecular and behavioral studies suggest that the α -Cre transgene does not alter the expression of adjacent chemosensory receptor genes.

Discussion

The α -Cre mouse is widely used in retinal research, e.g.^{3,5,6,33-36}. However, the integration site was unknown, precluding the development of genotyping primers from defining zygosity. It has also been unclear whether the transgene disrupts the expression of flanking genes. Previous work in our lab implied that α -Cre might lie on chromosome 7⁷. Indeed, here we mapped the insertion site of the α -Cre transgene to this chromosome using TLA²¹, a valuable method to map integration sites¹⁸⁻²⁰. The α -Cre transgene maps to 7qD3, in a cluster of *Vmn2r* GPCR genes, with loss of a 27 kb stretch. Relative to transcriptional direction, we found repetitive sequence upstream of the transgene, precluding the use of primers in this region to define zygosity. However, the downstream sequence was not repetitive, and we designed primer

sets that successfully distinguished hemi- or homozygous animals. These tools will be helpful to labs working with α -Cre mice. Further study revealed there are four copies of α -Cre transgene in tandem. Where the 3' end of the tandem copies meet chromosome 7 (the “left” end of the transgene), the SV40 poly A sequence is missing and terminates with the *Hbb* intron. We are confident that all four copies are inserted at a single genomic site as TLA revealed only a single insertion site.

Transgene insertion can affect the expression of nearby endogenous genes¹⁸. In many cases, insertion mutates an endogenous gene, leading to severe defects or lethality³⁷. However, there are no annotated genes in the α -Cre integration site. Nevertheless, transgenes can also integrate into endogenous regulatory elements or cause deletions and rearrangements that encompass such elements, and they may also indirectly alter chromatin structure^{18,38–40}. Importantly, we did not detect changes in the mRNA levels of seven olfactory-related genes flanking the α -Cre transgene, either in the nasal mucosa or retina. Moreover, a behavioral test did not detect any effect on olfactory function. Thus, we conclude that the α -Cre transgene has no significant effects on the expression of nearby endogenous genes.

The location of the transgene in a large cluster of olfactory genes led us to ask whether it is expressed in the olfactory system. The original α -Cre studies, based on GFP expression and Cre-dependent hPLAP/X-gal staining of embryos, did not report expression in non-retinal tissues^{3,4}. Using two different Cre reporter systems, we confirmed that the temporal and spatial pattern of expression of α -Cre in our mice matched that of the prior studies^{3,4}. We also found that α -Cre expression starts from E16 in post-mitotic AP2a⁺ amacrine cells all over the retina (including the central retina) without any gradient. This expression is not silenced and is persistent in adult mice⁵. Thus the expression of the α -Cre transgene in amacrine cells was much later than in retinal progenitors and may reflect the birthdate of these amacrine cells^{41,42}. In addition to the classic retinal pattern, we found α -Cre activity in two murine olfactory subsystems, the MOE and VNO. The VNO exhibited more positive cells, which is consistent with the proximity of the transgene to *Vmn2R* 68-69. The closest *Olfr* genes are *Olfr290* and *Olfr310*, which are 0.3Mb and 0.85Mb from the α -Cre transgene, respectively. MOE olfactory sensory neurons (OSNs) generally only express one *Olfr* gene per cell²⁹, which may explain why only a few OSNs were positive for α -Cre activity.

In summary, our results provide detailed information on the integration site of the α -Cre transgene on chromosome 7, the internal sequences retained during integration, and expression in both the developing retina and olfactory system. Primers from the left integration junction are suitable for distinguishing heterozygotes from homozygotes. These results will aid future application of α -Cre mice in the retina and olfactory system research.

Material And Methods

Mouse strains and genotyping

Mice were treated according to institutional and national guidelines. All animal procedures were reviewed and approved by the Animal Care Committee (ACC) of University Health Network (UHN), Toronto, Ontario, Canada (AUP#1411), and the Ethical Review Committee of Animal Research of West China Hospital, Sichuan University, Chengdu, Sichuan province, China (AUP# 2018008A), and performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and visual research. α -Cre mice (P Gruss), R26R mice (the Jackson Laboratory, stock 003474), Ai14 mice (the Jackson Laboratory, stock 007914) were housed in the Animal Resources Centre of UHN (Toronto, Canada) and the Laboratory Animal Center of Sichuan University (Chengdu, China) with a normal 12-h/12-h light/dark schedule. Fresh water and rodent diet were always available. Age-matched wild type ICR mice were used as α -Cre negative (No Cre) control. Genotyping was performed as before^{6,43} and as per The Jackson Laboratory guideline, and primers are listed in Table 3. Timed mating was set up according to the Jackson Laboratory's guide, and the day a vaginal plug was detected was considered as E0 of the pregnancy.

Table 3
Primers for genotyping

Mouse	Primer	Sequence	WT product	Transgene product
<i>α-Cre</i>	Cre-F	ATGTCCAATTTACTGACCG		724bp
	Cre-R724	CCCGGCCAAAACAGGTAGTTA		
R26R	R1295	GCGAAGAGTTTGTCTCAACC	550bp	250bp
	R523	GGAGCGGGAGAAATGGATATG		
	R26F2	AAAGTCGCTCTGAGTTGTTAT		
Ai14	oIMR9020	AAGGGAGCTGCAGTGGAGTA	297bp	200bp
	oIMR9021	CCGAAAATCTGTGGGAAGTC		
	oIMR9103	GGCATTAAAGCAGCGTATCC		
	oIMR9105	CTGTTCTGTACGGCATGG		

Targeted locus amplification (TLA) and sequence alignment

Spleen cells of α -Cre mice at P8 were isolated and prepared as previously described²¹. Samples were stored at -80°C until shipment to Cergentis (Utrecht, Netherlands) for TLA processing. TLA was performed as previously described²¹. In brief, cells were crosslinked by formaldehyde and cross-linked genomic DNA is digested into ~0.2 kb small fragments by restriction enzyme NlaIII (recognition sequence CATG) and ligated to form larger circular DNA. These DNA fragments are de-crosslinked and digested by NspI (recognition sequence RCATGY) to create ~2 kb fragments, which are then subjected to PCR with primers derived from sequences unique to the Cre transgene.

Primer set 1

RV461: 5'-ATTACGTATATCCTGGCAGC-3'

FW907: 5'-GGAGTTTCAATACCGGAGAT-3'

Primer set 2

RV330: 5'-AACCAGTGAAACAGCATTG-3'

FW408: 5'-GTTCGAACGCACTGATTTTC-3'

The PCR products were purified, and library prepped using the Illumina NexteraXT protocol and sequenced on an Illumina Miseq sequencer. Reads were mapped using Burrows-Wheeler Aligner's Smith-Waterman (BWA-SW). This allows partial mapping which is optimally suited for identifying break spanning reads. Version Mm10 was used for mapping the mouse genome.

Sequence Validations

Since breakpoint-spanning reads were available, two breakpoints and five linkers of the α -Cre transgene were confirmed by PCR amplification and Sanger sequencing. Primers were designed using Primer3 software (for primer sequences, see Table 2).

Different primer combinations were tested by gel-electrophoresis. PCR products with optimal results were sent to Sangon Biotech (Shanghai, China) for Sanger sequencing.

Genotyping of zygosity

Based on the results of TLA and PCR validation, we developed several genotyping assays for the zygosity differentiation of α -Cre (Table 1, Figure 2A). One assay included three primers: a shared forward genomic primer and two reverse primers designed specific to either the α -Cre or the wild-type allele.

Copy Number of transgenes

Tail genomic DNA was isolated from 26 mice with homozygote α -Cre, 38 mice with heterozygote α -Cre and 13 mice without α -Cre (wild type control). Real-time quantitative PCR using qTOWER 2.2 PCR machine (Analytik Jena) or C1000 touch Thermal Cycler (Bio-Rad) was employed to measure the relative level of a 143bp DNA fragment in Pax6 P0 promoter. Primers used are:

P0P F171: 5'-GGAGGACAATACCAGCCAGA-3'

P0P R312: 5'-GTTTCAGCTCGGCAGATTAGC-3'

Tests were run in duplicate with EvaGreen PCR Supermix (SsoFast, Bio-Rad) or SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific). PCR consisted of 40 cycles of denaturation at 95°C

for 15 seconds and annealing and extension at 55°C for 30 seconds. An additional cycle (95°C, 15 seconds) generated a dissociation curve to confirm a single product. Target gene relative expression was quantified according to the comparative Δ CT method. The signal levels were normalized against the housekeeping gene β -actin, and the average copy number of the Pax6 P0 promoter of wild type mice was set as 2. Then the copy number of the Pax6 P0 promoter of α -Cre mice was calculated based on the relative signal levels. The copy number of α -Cre transgenes was the average copy number of Pax6 P0 promoter minus 2.

Immunofluorescence and X-gal staining

Eyeballs or heads were fixed for 1 hour at 4°C in 4% paraformaldehyde, embedded in OCT (TissueTek 4583), frozen on dry ice and cut into 12-14 μ m sections on Superfrost slides. The following antibodies were used: Ap2a (Santa Cruz SC-8975), Ki67 (BD science Pharmingen 550609), GFP (Abcam ab5450). Antigen retrieval was performed as described ⁴³. Primary antibodies or labeled cells were visualized using donkey anti-mouse, donkey anti-rabbit antibodies conjugated with Alexa-488, Alexa-568 or Alexa-647 (1:1000; Molecular Probes). Nuclei were counter-stained with DAPI (Sigma, D9542) and mounted with Mowiol. Validations of the primary antibodies are provided on the manufacturers' websites or in the referenced citations.

For whole-mount *α -Cre*, *Ai14* mouse retinal staining, eyeballs were enucleated and incubated for 30 minutes in 4% paraformaldehyde. The retinas were incubated DAPI in PBS for 30min. X-gal (β -galactosidase) staining protocol is from Soriano Lab ²⁵. Retinal sections and whole-mount retinas of *α -Cre*, *R26R* mice were incubated with X-gal staining solution (1mg/ml X-Gal, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 in PBS) in dark for 2h at 37°C. After briefly washes with PBS, radial cuts were made to divide the retina into four quadrants to flatten the retina, and flat retinas and retinal section slides were mounted with Mowiol. Stained sections and slides were analyzed using a Zeiss Axio Imager Z2 fluorescence microscope and Zeiss Zen software. Color images were taken using an Olympus BX61 microscope.

Rna Extraction, Reverse Transcription And Quantitative Real-time Pcr

Total RNA was isolated from P13 nasal mucosa and retinas using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) followed by digestion with RNase-free DNase (DNA-free, Invitrogen, Thermo Fisher Scientific) to remove DNA contamination. Integrity and quantity of the RNA was evaluated by a NanoDrop (NanoDrop Technologies). Reverse transcription PCR assays were performed using a RT Reagent Kit with gDNA Eraser (TaKaRa). Real-time quantitative PCR using qTOWER 2.2 PCR machine (Analytik Jena) was employed to measure the expression level of seven genes. Primers used are listed in Table 4. Tests were run in duplicate on 3 separate biological samples with SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific). PCR consisted of 40 cycles of denaturation at 95°C for 15 seconds and

annealing and extension at 55°C for 30 seconds. An additional cycle (95°C, 15 seconds) generated a dissociation curve to confirm a single product. Target gene relative expression was quantified according to the comparative Δ CT method. Transcription levels were normalized against the housekeeping gene β -actin.

Table 4
Primers for RT-PCR of olfactory genes on Chromosome 7

Genes	Forward primers	Reverse primers
vmn2r67	accatagcaccaaaggcaag	tatagcatttgggggcaaaa
vmn2r68	gccacatcatcattgtttgc	cctggccaagaaagctacag
vmn2r69	tggatacatgggctctgttg	aagagcttggcctcattgaa
vmn2r70	gccacatcatcattgtttgc	cctggccaagaaagctacag
Olfr 291	tgcgctatccactctttgtg	gtcacaatggccaagaggat
Olfr 310	ccttgtcattgcaagtgtgc	tcagcaaagcaggaagatca
OMP	ctgcagttcgatcactggaa	cgtgtcatgaggttggtgag

Olfactory Function And Mobility Measurements

Mouse olfactory function and mobility were measured by the buried food pellet (BFP) test as previously described^{31,32,44}. Animals were tested at ~4 weeks of age. The mice were kept in individual cage (home cage) and food deprived overnight with access to water ad libitum. Mice were habituated for 30min by placing their home cage in the testing room. Then mice were introduced into a clean habituation cage (470×312×260mm) for 10min. This habituation step was repeated for three times with a new habituation cage each time. For the third time, the habituation cage was filled with 3cm-thick fresh corn-cob bedding. Then, the mouse was transferred back to its home cage for 10min and ready for test.

The test cage (size 470×312×260mm) was filled with 3-cm thick fresh bedding. A standard lab diet pellet (weight 1±0.1g) was buried approximately 0.5cm under the surface of the bedding. Set a timer for 5 min. The mouse to be tested was re-introduced into the test cage with head facing the cage wall. Time from the introduction of the animal in the cage until it retrieved the food pellet with its front paws was measured in seconds up to a maximum of 300s (5 min). After each test, bedding was changed, and test cage were cleaned with animal room cleaning solution. One mouse was tested for three times with 10-min intervals in its home cage. The sites of animal placement were always the same, but the location of the food pellet was changed for each test in a random fashion.

To assess the mobility of mice, the test cage was divided into four equal quadrants and each movement of the mouse from one quadrant into a neighboring quadrant was scored, whenever more than half of its

body length (excluding the tail) had entered the new quadrant. Mobility levels were scored as quadrants/s.

Statistical analysis

All data are expressed as the means $\pm$ SD. Statistical analyses were performed using SPSS version 26.0. The differences between mean values were evaluated using a one-way ANOVA test. A P-value less than 0.05 was considered to indicate a statistically significant difference between means.

Declarations

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Authors' Contributions

Conception and design: DC and RB. Analysis and interpretation: YF, WC, RW, DC, RB. Data collection: YF, WC, RW, WQ, JP, TY, DC. Obtain funding: DC and RB. Overall responsibility: DC and RB. Manuscript writing and editing: YF, WC, RW, DC, RB. All authors read and approved the final manuscript.

Declaration of competing interest

No conflicting relationship exists for any author.

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Figures

Figure 1

α -Cre locus identification. (A) Schematic of the vector used to make α -Cre mice (including Pax6 α -enhancer, Pax6 P0 promoter, Cre, IRES, GFP, Hbb intron, and SV40 poly A sequence), showing positions of primers within Cre used for TLA. (B) TLA sequence coverage across the mouse genome using primer set 1. The chromosomes are indicated on the y-axis, the chromosomal position on the x-axis. Similar results were obtained with primer set 2. Peak at Chromosome 7 shows inserted sequence (black circle). (C) Regional coverage of the α -Cre insertion site on Chromosome 7 using both sets of primers, showing a 27 kb deletion and spanning 2 Mb. Some RefSeq genes flanking the insertion site are also indicated.

Figure 2

Confirmation of the transgene insertion site on Chromosome 7. (A) Map of the transgene/chromosome 7 left and right junctions. Primers were designed to confirm the left and right junctions between the transgene and Chromosome 7, as well as the predicted deletion. (B) Primers 1 and 2 were used to confirm the 27 kb deletion. The 250 bp band is absent in putative homozygous α -Cre animals. Primers 1 and 5 were used to confirm the left junction between the 3' end of the α -Cre transgene and the 5' end of Chromosome 7 in homozygous animals. This 722 bp band is absent in wild-type animals. (C) The chromatogram of the 722 bp band in (B) showing the chromosome 7 sequence linked to the Hbb intron through an 18bp linker. No intervening SV40 poly A sequence was detected. (D) Primers 3 and 4 were designed to confirm the right-hand junction. However, the 250 bp band is still present in putative homozygous α -Cre animals (arrow). Primers 4 and 6 were used to confirm the right junction between the 5' end of the α -Cre transgene and the 3' end of Chromosome 7 in homozygous animals. This 436 bp band is absent in wild-type animals. (E) The 500 bp chromosome 7 sequence at the right junction of the α -Cre transgene is repetitive. Four such repeats are indicated around Vmn2r67 and Vmn2r68, (red arrows).

Figure 3

Evidence for four tandem transgene copies. (A) Sequences from various PCR fragments were stitched together to deduce the arrangement of the α -Cre transgene, including the linker segments 1-5, but missing the SV40 polyA tail. The 1465 bp α enhancer shown here is adjacent to the 3' side of chromosome 7. The position of the SV40 polyA tails and other tandem copies of the transgene were deduced in (B-H). (B) Schematic showing the predicted junction between two tandem transgenes; the vertical dashed line indicates the junction site. The indicated primers were used to deduce the relationship between SV40 polyA signal and the Hbb intron or α -enhancer. (C) Primers from the Hbb intron (F66, F36) and SV40 polyA signal (SV40-1F, -2F, -R) confirmed that the SV40 signal is downstream of the Hbb intron. (D) Primers from the α -enhancer (R351) and SV40 polyA signal (SV40-1F, -2F, -R) confirmed the existence of tandem transgene copies. Blue arrows indicate multiple bands with R351/SV40-1F, but only one band with R351/SV40-2F primers. (E) Primers from Hbb intron (F36) and the α -enhancer (R351) confirmed three versions of the SV40/ α -enhancer junction. (F) Schematic, based on sequencing of the PCR products in (E) showing the relationship between long, medium and short versions of the SV40/ α -enhancer junction. Dashed boxes (48bp or 281bp) indicate the deleted regions. (G) Quantitative PCR was used to measure transgene copy numbers in WT mice versus those that were heterozygous or homozygous for α -Cre. (H) Schematic showing 4 tandem copies of the α -Cre transgene in the chromosome 7 insertion site. The dashed boxes highlight the regions shown in more detail in (B) and (F). Note that only 3 copies include the SV40 polyA signal, and the α enhancer is a slightly different size in each copy (See sizes in (F)). Apart

from the right-most copy, the order of these variants along the chromosome is undetermined. (I) Schematic showing α -Cre location relative to the entire chromosome and select chromosome 7 loci (top), and the neighboring *Vmn2* and *Olfr* clusters within 7qD3 (bottom, dashed orange box). *Hbb*, hemoglobin beta gene; L5, linker 5; OMP, Olfactory marker protein gene; V1, V2 vector sequence 1 and 2 flanking the SV40 polyA signal; *Vmn1r*, vomeronasal receptor 1; *Vmn2r*, vomeronasal receptor 2; *Olfr*, olfactory receptor.

Figure 4

α -Cre activity in the retina. (A) Horizontal sections of α -Cre;Ai14 head at E12, showing tdTomato in the nasal and temporal peripheral retina as well as RGC axons in the optic nerve and optic chiasm. (B) X-gal staining of α -Cre;R26R eyeball at E14, showing α -Cre activity in the nasal and temporal peripheral retina. (C, D) X-gal staining of α -Cre;R26R whole retina at P0 (C), and tdTomato fluorescence in α -Cre;Ai14 at P18 both showing α -Cre activity in the nasal and temporal peripheral retina but not the dorsal and ventral retina. (E) Horizontal sections of α -Cre;Ai14 retina at E12, showing α -Cre expression (anti-GFP immunofluorescence) and lineage tracing (tdTomato fluorescence) in the nasal and temporal peripheral retina. (F) Horizontal sections of α -Cre;Ai14 retina at E16, showing GFP in amacrine cells (white arrows). (G) AP2a staining (red) of P8 α -Cre retina, white arrows show typical GFP+;AP2a+ cells. (H) Ki67 staining (red) of E16 α -Cre retina; white arrows show typical GFP+;Ki67- cells ON, optic nerve; ONL, outer nucleus layer; INL, inner nucleus layer; GCL, ganglion cell layer; NBL, neuroblastic cell layer. Scale bars: (A, B) 200 μ m; (C, D) 50 μ m.

Figure 5

α -Cre activity in the olfactory system. (A-D) Horizontal sections of E16 α -Cre;Ai14 head showing α -Cre expression (anti-GFP) and lineage tracing (tdTomato) in the retina (white arrows) and the VNO (dotted box) which is magnified in (B-D). (E-H) Horizontal sections of E16 α -Cre;Ai14 head showing α -Cre expression (anti-GFP) and lineage tracing (tdTomato) in the retina, optic nerve, optic chiasm, lateral geniculate body (white arrows) and nasal olfactory epithelium (dotted box) which is magnified in (F-H). Green arrows indicate tdTomato+ olfactory sensory neurons. (I-L) Sections of P0 α -Cre;Ai14 head showing α -Cre expression (anti-GFP) and lineage tracing (tdTomato) in olfactory epithelium, olfactory nerve (white arrow) projecting to olfactory bulb (I-J), as well as in vomeronasal nerve (white arrow) projecting to AOB (K-L). GFP, green fluorescent protein; VNO, Vomeronasal organ; OB, olfactory bulb; AOB, accessory olfactory bulb. Scale bar: 50 μ m.

Figure 6

Effects of the α -Cre transgenes on olfactory gene expression and function. (A) RT-PCR was used to assess expression of the indicated genes from nasal mucosa (NM) and retina of indicated genotypes at P13. (B-C) The mobility score (B) and the latency to find buried pellets (C) of the indicated genotypes at P30. Error bars represent SD of measurements and asterisks indicate significant differences between indicated genotypes (* $p < 0.05$, ** $p < 0.01$, one-way ANOVA followed by Bonferroni correction).