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Transgenic Cre recombinase expression in germ cells and early embryogenesis directs homogeneous and ubiquitous deletion of loxP-flanked gene segments

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Abstract We report on the establishment of a transgenic mouse line expressing Cre recombinase under control of the c-kit promoter. Expression of Cre recombinase was only observed in late spermatogenesis and oogenesis, however, Cre-mediated deletion of floxed gene segments occurred at this stage as well as in early embryogenesis. As a consequence of this, a chimeric distribution of loxed alleles was found in a large fraction of these mice. The chimerism was very homogeneous in different organs and tissues of the same individual but varied between different individual offspring. The potential uses for this mouse line are discussed.

Key words: Transgenic mouse; Cre recombinase; Embryogenesis; Germ cell

1. Introduction

The possibility to generate defined mutations in embryonic stem (ES) cells by gene targeting has advanced the knowledge of gene function in mammals immensely [1]. Generally, a mutation is introduced into the genome of ES cells by homologous recombination followed by injection of the cells into mouse blastocysts. In this manner chimeric mice can be generated enabling the establishment of mouse lines carrying the defined mutation. A drawback of this technique is that mutation of a gene in the germline frequently results in embryonic lethality [2], making studies of gene function at later developmental stages impossible. Other limiting factors include the fact that germline mutations can cause complex pleiotropic phenotypes difficult to analyze. Redundant gene products can also compensate for the mutation, concealing possible phenotypic consequences. These limitations all prevent functional studies of a given gene product in defined tissues, or cell subsets in the organism, from the developing embryo to the adult mouse.

A strategy to overcome the limitations in conventional knock-out systems has been developed, based on the Cre-loxP recombination system [3]. Cre recombinase originating from the bacteriophage P1 mediates site-specific recombination between specific 34-bp repeats, termed loxP sites. A loxP flanked (floxed) DNA segment can be deleted upon Cre recombinase delivery, leaving a single loxP site behind. Inactivation of loxP targeted genes can be restricted to specific tissues or cell lineages by introducing the Cre recombinase under the control of a cell type specific promoter [4]. Activation of transgenes by Cre-mediated deletion of sequences that inhibit transcription [5] and inducible Cre expressing systems [6] have been described. To establish mouse lines with a restricted expression of the Cre recombinase, we generated transgenic (TG) mice expressing Cre recombinase under control of the c-kit promoter. The c-kit promoter has been shown to be active in a large array of tissues including cells of hematopoietic origin [7], CNS [8], melanocytes, testis, ovary [9], and early embryogenesis [10]. Here we describe a transgenic mouse line functionally expressing Cre uniquely in late stages of spermatogenesis/oogenesis and in early embryogenesis.

2. Materials and methods

2.1. Cre expression vector and generation of transgenic mice

The c-kit promoter [11] was amplified by PCR of genomic DNA from a C57BL/6 mouse using the following primers: Fwd: 5′-CCC AAG CTT AGG GAG AGG TGC TAG-3′; Rev: 5′-CCG CTC GAG TGC GCT AGA CTC TGA-3′.

The 300-bp fragment containing the promoter was digested with *Hin*dIII and *Xho*I, and inserted into a vector comprising the Cre cDNA and the 3′-UTR of the MT-I gene containing the poly(A) signal (kindly obtained from Dr. F. Alt [12]) (Fig. 1A). The promoter was sequenced and verified against the published sequence [11]. The construct was gel purified and injected into pronuclei of fertilized oocytes of F1(C57BL/6×CBA) mice. The injected zygotes were transferred to pseudopregnant female mice by standard procedures. Transgenic mice were detected by preparation of genomic tail DNA which was subjected to Southern blot hybridization (previously described in [13]) using a 0.4-kb probe complementary to the Cre cDNA (Fig. 1A,B). Six founder mice were obtained and used to establish transgenic lines by backcrossing to C57BL/6 mice. In this study, the characterization of mice from the c-kit (143) Cre line is reported.

2.2. Detection and quantification of functional Cre recombinase expression

Transgenic mice were crossed to a mouse strain harboring a reporter gene (neomycin resistance gene) flanked by loxP sites (I.B., unpublished) (Fig. 2A). Genomic DNA from mice expressing transgenic Cre recombinase and containing a floxed neomycin resistance (Neo) reporter gene segment was prepared from various organs using a Nuclon st for soft tissue kit (Amersham, UK). Ten μg of DNA was run on a 0.85% agarose gel and blotted onto a Zeta-Probe GT membrane (Bio-Rad, USA). The filters were hybridized and washed according to manufacturers' recommendations. The fragment used as a probe to detect Cre-mediated recombination was a 0.6-kb *Xba*I/*Hpa*I fragment hybridizing to a region 3 kb 3′ of the Neo gene (Fig. 2A). The intensity of the three bands representing wild-type (10.1 kb), floxed (6.2 kb) and loxed (13.0 kb) alleles (Fig. 2) was determined using a Phosphor Imager (Molecular Dynamics, USA). The integrated volume of the bands was quantified by standardized rectangular boxes using Image Quant software version 3.3 (Molecular Dynamics). All calculations were corrected for lane background. Deletion index for all organs was determined by comparing the signal intensities of the bands representing the three alleles. The deletion index was calculated as follows: deletion index in % = intensity of loxed allele/(intensity of loxed allele+intensity of floxed allele)×100. As an internal control the ratio of intensity of loxed allele/intensity of wild-type allele×100 was

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determined. The control should equal the deletion index. For technical reasons a deviation of 5% was allowed. All data presented is based on samples meeting this criteria.

2.3. Histochemical analysis of functional Cre expression

Transgenic mice from the c-kit (143) Cre line were crossed to cAct-XstopXlacZ transgenic mouse strain (kindly provided by Dr. D.J. Anderson) [14]. Offspring were genotyped by Southern blot hybridization of genomic DNA from tail tips. Probes used to detect double transgenic mice were the 0.4-kb Cre probe described above and a 3-kb probe covering most of the lacZ gene. Double transgenic, 4 week old females were injected intraperitoneally with 5 U of Pregnant Mares' Serum (PMS) (Sigma, USA), and the ovaries were dissected out 46 h thereafter. Testes from double positive transgenic mice were dissected from 8 week old mice. Dissected organs were rapidly frozen in mounting media (Tissue-Tek OCT 4583 Compound; Sakura Finetek, The Netherlands). Cryosections (20 μm) were prepared and fixed 2 min in 99.5% ethanol. The fixed sections were subjected to X-gal staining overnight at 37°C. The X-gal staining contained 1 mg/ml X-gal (4-chloro-5-bromo-3-indolyl-β-galactosidase), 4 mM $K_4Fe(CN)_6$, 4 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS. The X-gal stained slides were washed twice in PBS and were counterstained with hematoxylin before microscopy.

3. Results and discussion

3.1. Construction and screening of transgenic mice expressing Cre recombinase under the control of the c-kit promoter

The construct used for the production of c-kit-Cre TG mice was generated by PCR amplification of the c-kit promoter [11] which subsequently was cloned into a vector comprising the cDNA encoding the Cre recombinase (Fig. 1A). The construct was injected into fertilized oocytes, and transferred to the oviducts of pseudo-pregnant female mice. Six founders were identified as positive for integration of the construct into the genome, as demonstrated by Southern blot hybridization (Fig. 1B).

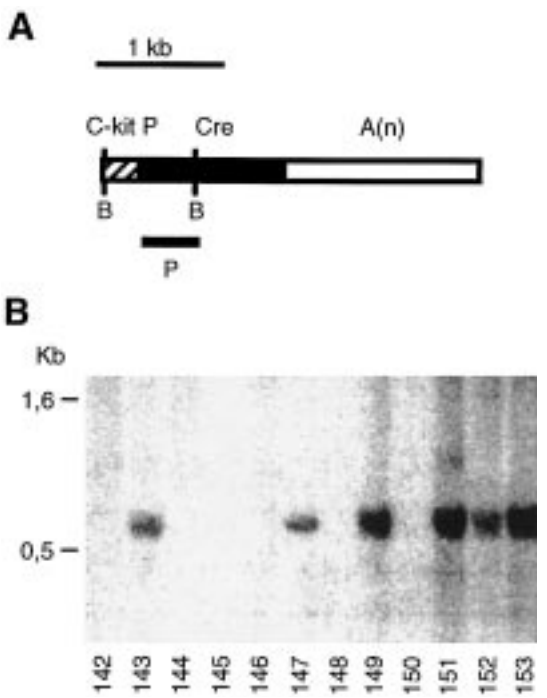

Fig. 1. A: Schematic representation of the construct used to generate c-kit Cre transgenic mice. The 0.3-kb fragment containing the c-kit promoter is represented by a striped box, the Cre coding sequence by a filled box and the 3′-UTR of the MT-I gene containing the poly(A) signal by an open box. Also shown is the 0.4-kb probe (P) used for genotyping. The *Bam*HI restriction enzyme sites are denoted B. B: Southern blot analysis of *Bam*HI-digested tail derived genomic DNA. Six founders were found to have the construct integrated into their genome when hybridized to the probe shown in A.

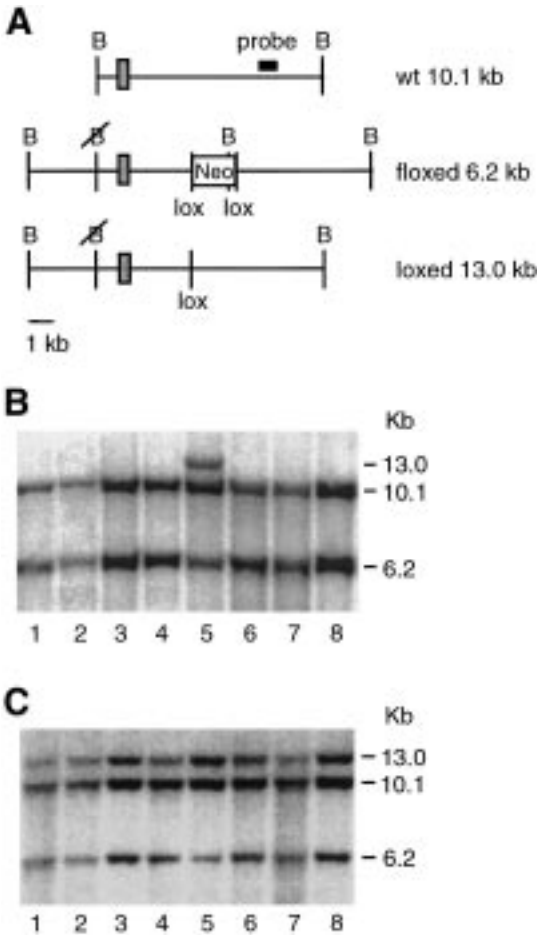

Fig. 2. A: Schematic representation of the targeted locus in the Neo reporter mouse strain: the wild-type locus (upper), the targeted allele with the two introduced loxP sites (middle) and the targeted allele after Cre-mediated depletion of floxed gene segment (lower). As a consequence of the construction of the targeting vector used to introduce the floxed Neo reporter gene, a *Bam*HI site was destroyed. This is indicated as a crossed-over *Bam*HI site. The sizes of the three possible fragments after *Bam*HI digestion are indicated. Wild-type allele: 10.1 kb; targeted allele: 6.2 kb; and the allele after Cre mediated deletion of the Neo gene: 13.0 kb. The probe used for hybridization is also indicated. B: Deletion of the floxed allele in testis. Southern blot analysis of genomic DNA from 8 different organs from a mouse carrying the Neo reporter gene and the c-kit (143) Cre TG. *Bam*HI-digested DNA was hybridized with the probe shown in A. The three bands represent the loxed (13 kb), wild-type (10.1 kb) and floxed (6.2 kb) allele in A. Tail (lane 1); liver (lane 2); brain (lane 3); kidney (lane 4); testis (lane 5); thymus (lane 6); spleen (lane 7); bone marrow (lane 8). C: Mosaic deletion of floxed allele. Southern blot analysis of genomic DNA from 8 different organs from a mouse carrying the Neo reporter gene and the c-kit (143) Cre TG. *Bam*HI-digested DNA was hybridized as in B. Tail (lane 1); liver (lane 2); brain (lane 3); kidney (lane 4); testis (lane 5); thymus (lane 6); spleen (lane 7); bone marrow (lane 8).

3.2. In vivo functional analysis of Cre recombinase activity

To test for the function of the integrated c-kit-Cre transgene, TG founders were back-crossed to C57BL/6 mice. Transgenic offspring were crossed to a mouse strain carrying

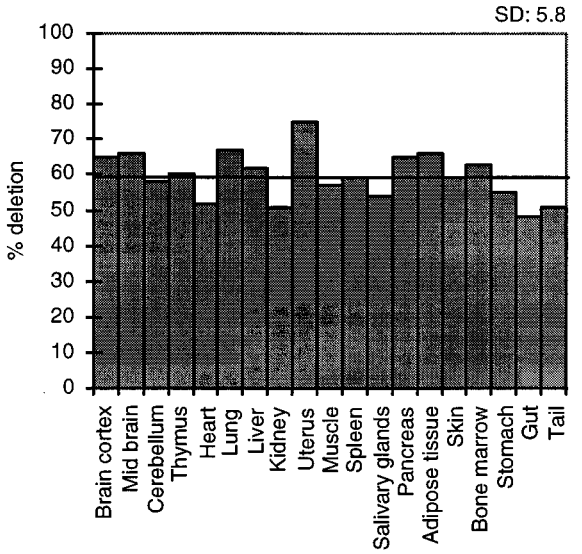

Fig. 3. Deletion index in different organs of a representative individual mosaic mouse carrying the c-kit (143) Cre TG together with the Neo reporter locus. Histograms show deletion index in 19 different organs indicated. Genomic DNA was prepared and hybridized as described (Section 2). Mean value of deletion index is indicated by a black line, and standard deviation of the mean is displayed at the top.

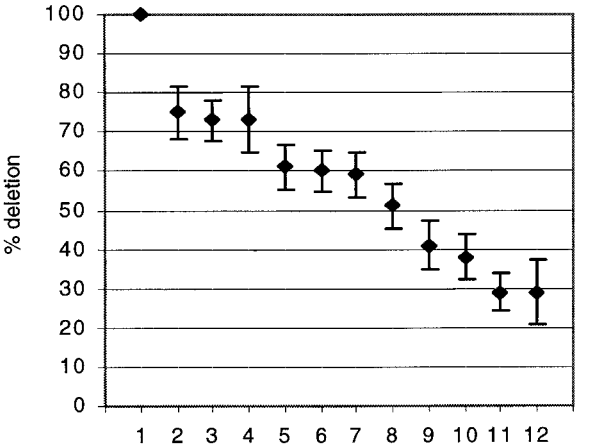

Fig. 4. Mean value of deletion index in individual mosaic mice carrying the c-kit (143) Cre TG together with the Neo reporter locus. Deletion index was calculated for 12–18 organs in 12 different mosaic individuals and the mean value plus standard deviation for each individual is shown. The highest standard deviation of the mean was 8.5 (mouse #3) and the lowest 4.8 (mouse #11). Deletion index for testis was not included in the analysis due to the additive effect of the non-mosaic and mosaic Cre expression pattern. Mouse #7 is identical to the mouse shown in Fig. 3.

a reporter Neo gene flanked by loxP sites (floxed) which had been introduced by gene targeting (Fig. 2A). Deletion of the reporter gene results in a restriction fragment length polymorphism (RFLP) of the genomic DNA. The floxed allele is represented by a 6.2-kb fragment and the deleted (loxed) allele by a 13.0-kb fragment.

To investigate the functional expression of Cre-recombinase, deletion of the floxed reporter gene was measured in different organs. The three bands representing floxed, loxed, and wild-type alleles were clearly visible and quantified by Southern blots (Fig. 2). Deletion index was determined as the intensities of the bands representing the loxed allele/(floxed allele+loxed allele)×100. In one of the transgenic lines obtained (c-kit (143) Cre) deletion of floxed gene segments was observed specifically in testis (Fig. 2B). Moreover, in a fraction (37%) of the mice deletion of the floxed reporter gene was also observed in a mosaic fashion in all other organs analyzed (Fig. 2C).

Deletion of the floxed sequence in germ cells was shown to be stably inherited (Table 1). Amongst 19 offspring carrying the reporter locus born from female c-kit (143) Cre TG reporter mice bred to male C57BL/6 mice, 12 individuals inherited a loxed allele of the reporter gene but not the c-kit (143) Cre TG while 7 individuals inherited both the c-kit (143) Cre TG and a loxed allele. Similarly, in 12 offspring carrying the reporter locus born from C57BL/6 females crossed to male c-kit (143) Cre TG reporter mice, 5 were found that had inherited the loxed allele but not the c-kit (143) Cre TG and 7 had inherited the c-kit (143) Cre TG together with a loxed allele of the reporter gene. In all these mice no inheritance of a floxed allele was detected. Together, these data demonstrate that the c-kit (143) Cre mouse deletes loxP flanked gene segments in male and female germ cell with a high efficiency.

To further characterize the mosaic phenotype observed in tail derived DNA from offspring of reporter×c-kit Cre (143) crosses, the degree of mosaicism was determined in an expanded set of tissues and organs (Fig. 3). Genomic DNA from 19 different tissues representing a large array of cell lineages of early embryonic development was prepared and subjected to Southern blot analysis. Whereas the degree of chimerism varied substantially between individuals (Fig. 4), the deletion index was shown to be consistent in all tissues analyzed within a given individual (Fig. 3).

3.3. Maternal effect on functional Cre expression

The deletion index observed in the mosaic individuals varied within a range of 20–100% (Fig. 4). In 13 individuals with no apparent mosaicism, the deletion index in testis was found

Table 1
Genetic analysis of germ cell specific Cre mediated recombination

Origin of female parent[a]	Origin of male parent	No. of offspring with Cre deletion in germ line[b]		No. of offspring without Cre mediated deletion in germ line	
		Cre TG$^-$	Cre TG$^+$	Cre TG$^-$	Cre TG$^+$
Neo floxed, Cre TG$^+$	B6	12	7	0	0
B6	Neo floxed, Cre TG$^+$	5	7	0	0

[a]Female mice carrying the floxed reporter gene and the Cre transgene were crossed to B6 male mice. Male mice carrying the floxed reporter gene and the Cre transgene were crossed to B6 female mice.
[b]Tails from offspring were analyzed for deletion of floxed reporter gene and inheritance of Cre transgene. Deletion of the floxed allele was either 0% or 100%. Figures indicate number of mice.

to vary between 25% and 49% (data not shown). Interestingly, the proportion of offspring displaying mosaic distribution of loxed alleles of the reporter gene was found to depend on whether the c-kit (143) Cre TG was maternally or paternally inherited. Thus, while 47% of the offspring from a c-kit (143) Cre TG female displayed a general mosaic pattern, only 23% of the offspring from a c-kit (143) Cre TG male displayed the general mosaic deletion of the floxed allele (Fig. 5). The fact that 23% of the offspring of male c-kit (143) Cre TG mice became mosaic suggested that the Cre-mediated deletion of the floxed reporter gene is in part due to the transcription of the Cre TG in the early embryo. However, the significantly higher frequency of mosaic among offspring of the c-kit (143) Cre TG females demonstrated a maternal effect reflecting the expression of Cre in the oocyte. No significant correlation between the degree of mosaicism and the maternal vs. paternal expression of the Cre TG could be detected (data not shown).

3.4. Specific expression of Cre in mature germ cells

To further investigate the functional expression of the Cre recombinase in testis, the c-kit (143) Cre mouse line was crossed to the transgenic mouse strain cAct-XstopXlacZ [14]. This strain carries the lacZ gene under control of the chicken β-actin promoter. A loxP flanked STOP sequence preceding the lacZ gene prevents transcription of the lacZ gene. Cre-mediated deletion of the STOP sequence renders the transgene transcriptionally active and the gene product, β-galactosidase, can be visualized by X-gal staining.

Non-mosaic male offspring from the c-kit (143) Cre×cAct-XstopXlacZ crosses were subjected to cryo-sections of the testis. Microscopy of the X-gal stained cryo-sections revealed that functional Cre expression was restricted to late spermiogenesis (Fig. 6A). A reproducible staining pattern corresponding to a specific expression in secondary spermatocytes, spermatides and spermatozoa was observed. However, spermatogonia, Sertoli cells and fibroblast cell layers were negative for X-gal staining. No background staining could be detected in Cre negative littermates (data not shown).

We next analyzed non-mosaic female offspring from the c-kit (143) Cre×cAct-XstopXlacZ crosses for functional expression of Cre in the ovaries. As demonstrated in Fig. 6B, lacZ expression was observed uniquely in oocytes of mature follicles 46 h after treatment of the females with PMS in around 10 oocytes per individual. No cells expressing lacZ could be detected in ovaries of untreated mice (data not shown). The failure to detect lacZ positive oocytes in untreated females could be due to a limited functional penetrance of the expressed Cre recombinase, together with the fact that PMS

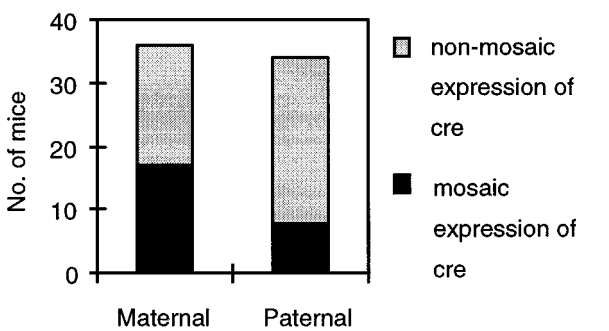

Fig. 5. Mode of inheritance of the c-kit (143) Cre TG influences Cre expression pattern. The frequencies of mosaic vs. non-mosaic Cre-mediated deletion of the Neo reporter gene in offspring of male or female c-kit (143) Cre TG mice. Seventeen out of 36 individuals (47%) displayed mosaicism when the transgene was maternally inherited, compared to 8 out of 34 (24%) when the transgene was paternally inherited.

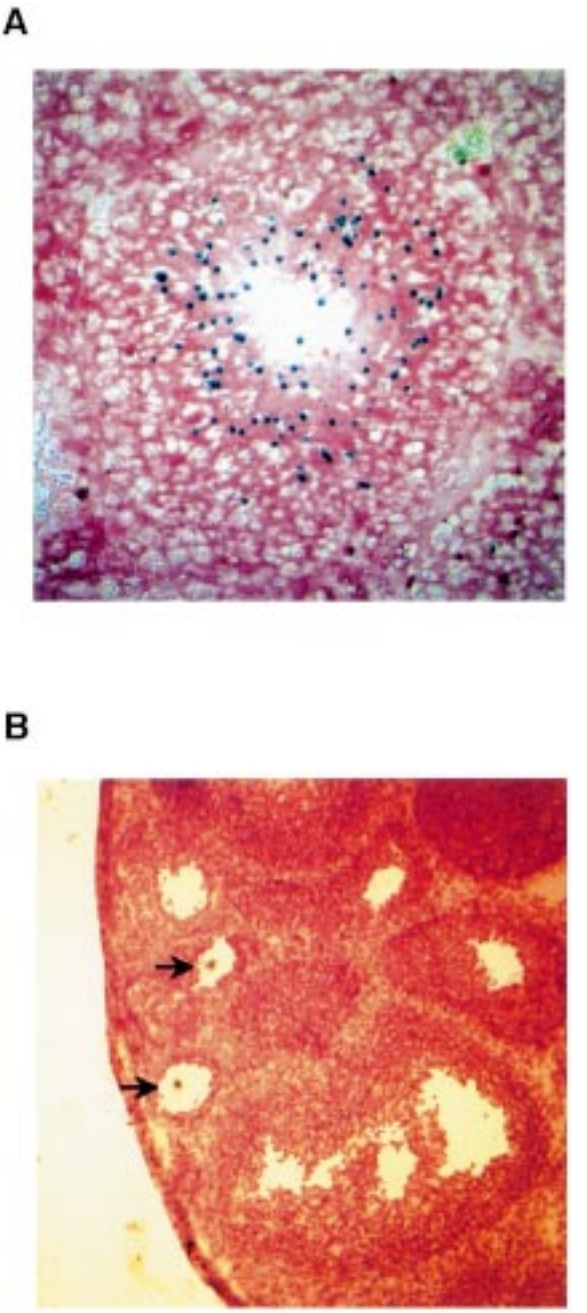

Fig. 6. Functional expression of Cre recombinase in mature germ cells of the c-kit (143) TG mouse. X-gal stained cryosection of testis (A) and an ovary (B) from non-mosaic mice carrying the cAct-XstopXlacZ TG together with the c-kit (143) TG. Only cells in which Cre-mediated depletion of the floxed STOP sequence has occurred stain positive as a result of rendering the transgenic lacZ gene transcriptionally active. A: In testis, only cells representing late stages of spermiogenesis scored positive. B: For staining of the ovary the mouse was pre-treated with PMS 46 h prior to sacrifice. Arrows indicate positively staining oocytes in two of the follicles shown.

treatment is known to induce the activation of 20–60 eggs while 8–12 eggs are released in normal ovulation [15]. No other cells were found to express lacZ with or without superovulation treatment (data not shown).

4. Concluding remarks

All individual chimeric c-kit (143) Cre mice display a homogeneous and generally distributed chimerism in various tissues of a given individual supporting the notion that functional Cre expression only occurs very early in development and not at later stages of development at least in the tissues analyzed here. This feature of the mouse line would make it a powerful tool for general screenings of the effect of loxP-Cre mediated deletion of specific gene segments, particularly of those with early lethal effects [2]. In comparison with other similar mouse lines [16] the c-kit (143) Cre appears to display a more homogeneous distribution of chimerism. Thus, this model provides a more sensitive experimental system when screening for gene function in multiple organs and tissues.

The functional Cre expression specifically demonstrated in late germ cell development and early embryogenesis of the c-kit (143) Cre mice constitutes an additional area in which this mouse model could prove useful, providing the possibility to study gene effects at these distinct stages of development.

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References

[1] Thomas, K.R. and Capecchi, M.R. (1987) Cell 51, 503–512.
[2] Copp, A.J. (1995) Trends Genet. 11, 87–93.
[3] Sauer, B. and Henderson, N. (1988) Proc. Natl. Acad. Sci. USA 85, 5166–5170.
[4] Gu, H., Marth, J.D., Orban, P.C., Mossmann, H. and Rajewsky, K. (1994) Science 265, 103–106.
[5] Lasko, M., Sauer, B., Mosinger Jr., B., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L. and Westphal, H. (1992) Proc. Natl. Acad. Sci. USA 89, 6232–6236.
[6] Kuhn, R., Schwenk, F., Aguet, M. and Rajewsky, K. (1995) Science 269, 1427–1429.
[7] Andre, C., d'Auriol, L., Lacombe, C., Gisselbrecht, S. and Galibert, F. (1989) Oncogene 4, 1047–1049.
[8] Qiu, F.H., Ray, P., Brown, K., Barker, P.E., Jhanwar, S., Ruddle, F.H. and Besmer, P. (1988) EMBO J. 7, 1003–1011.
[9] Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A. and Besmer, P. (1989) Genes Dev. 3, 816–826.
[10] Orr Urtreger, A., Avivi, A., Zimmer, Y., Givol, D., Yarden, Y. and Lonai, P. (1990) Development 109, 911–923.
[11] Yasuda, H., Galli, S.J. and Geissler, E.N. (1993) Biochem. Biophys. Res. Commun. 191, 893–901.
[12] Xu, Y., Davidson, L., Alt, F.W. and Baltimore, D. (1996) Immunity 4, 377–385.
[13] Tornberg, U.C., Bergqvist, I., Haury, M. and Holmberg, D. (1998) J. Exp. Med. 187, 703–709.
[14] Tsien, J.Z. et al. (1996) Cell 87, 1317–1326.
[15] Wassarman, P.M. and Kinloch, R.A. (1992) Mutat. Res. 296, 3–15.
[16] Betz, U.A., Vosshenrich, C.A., Rajewsky, K. and Muller, W. (1996) Curr. Biol. 6, 1307–1316.