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ORIGINAL PAPER

# Mast cell-specific Cre/loxP-mediated recombination in vivo

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**Abstract** Mast cells are important effectors of type I allergy but also essential regulators of innate and adaptive immune responses. The aim of this study was to develop a Cre recombinase-expressing mouse line that allows mast cell-specific inactivation of genes in vivo. Following a BAC transgenic approach, Cre was expressed under the control of the *mast cell protease (Mcpt) 5* promoter. *Mcpt5-Cre* transgenic mice were crossed to the ROSA26-EYFP Cre excision reporter strain. Efficient Cre-mediated recombination was observed in mast cells from the peritoneal cavity and the skin while only minimal reporter gene expression was detected outside the mast cell compartment. Our results show that the *Mcpt5* promoter can drive Cre expression in a mast cell-specific fashion. We expect that our *Mcpt5-Cre* mice will be a useful tool for the investigation of mast cell biology.

**Keywords** Mast cells · Conditional gene targeting · Cre/*loxP*-mediated recombination · Cre transgenic mice

**Abbreviations**

| | |
|---|---|
| BAC | Bacterial artificial chromosome |
| BMMC | Bone marrow-derived mast cells |
| EYFP | Enhanced yellow fluorescent protein |
| FACS | Fluorescence activated cell sorting |
| floxed | *loxP*-flanked |
| kb | Kilobases |
| Mcpt | Mast cell protease |
| HR | Homology region |
| MACS | Magnetic activated cell sorting |
| neo | Neomycin resistance gene |
| SCF | Stem cell factor |

## Introduction

Mast cells are central effector cells in type I allergy (Kawakami and Galli 2002; Metcalfe et al. 1997), but have recently been shown to be important initiators

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and effectors also of innate immunity as well as modulators of adaptive immune responses (Galli et al. 2005a, b; Marshall and Jawdat 2004). Many reports demonstrated a role of mast cells in host defence against pathogens (Dawicki and Marshall 2007; Echtenacher et al. 1996; Malaviya et al. 1996) and implicated a function of these cells in wound healing, tissue remodeling and transplant tolerance (Lu et al. 2006; Trautmann et al. 2000; Weller et al. 2006). A pathogenic role of mast cells was discussed in rheumatoid arthritis, systemic sclerosis, multiple sclerosis and atherosclerosis (Bachelet et al. 2006; Irani et al. 1992; Lee et al. 2002; Secor et al. 2000; Sun et al. 2007).

Investigation of mast cell biology was severely hampered by the scarcity of these cells in tissues. Experimental systems that have widely been used are in vitro analysis of mast cell lines or mast cells differentiated in vitro from bone marrow (BMMC). In vivo analysis of the function of genes in mast cells until today relied on the reconstitution of mast cell-deficient rodents with mast cells derived from bone marrow or hematopoietic cells of gene-deficient animals or from gene-deficient embryonic stem cells (Kitamura et al. 1978; Nakano et al. 1985; Tsai et al. 2002, 2000). These systems yielded valuable information, but suffer important limitations. In the mouse lines $kit^{W/W\text{-}v}$ and $kit^{W\text{-}sh/W\text{-}sh}$, which are commonly used for reconstitution experiments, spontaneous mutations of the *kit* gene coding region or its regulatory elements result in reduced activity of the kit receptor that controls development and survival of mast cells (Galli et al. 2005b; Grimbaldeston et al. 2005). In addition to mast cell-deficiency, $kit^{W/W\text{-}v}$ animals are characterized by sterility, anemia, absence of melanocytes and interstitial cells of Cajal, a high incidence of dermatitis, papillomas of the forestomach, gastric ulcers and dilation of the duodenum (Galli et al. 2005b). $kit^{W\text{-}sh}$ animals, which lately are being used instead of $kit^{W/W\text{-}v}$ mice, seem to feature a more narrow defect with deficiency for mast cells, melanocytes, and interstitial cells of Cajal (Grimbaldeston et al. 2005). The transfer of *in vitro* cultivated mast cells into mast cell-deficient animals by intradermal, intraperitoneal or intravenous injections can result in mast cell numbers, which, dependent on the route of cell transfer and the anatomical site, can approach normal mast cell density, but often remain considerably lower with significant variability (Grimbaldeston et al. 2005; Tsai et al. 2005).

The Cre/*loxP* recombination system represents a powerful tool that allows for conditional, e.g. cell type-specific, inactivation of genes in the mouse (Rajewsky et al. 1996). Since the first description of in vivo mutagenesis using the Cre/*loxP* system (Gu et al. 1994), an impressive number of floxed mouse lines has been generated. Herein, we report the first *Cre* transgenic line allowing mast cell-specific gene inactivation in vivo.

## Material and methods

### Construction of the transgene and generation of transgenic mice

The construct was based on a bacterial artificial chromosome (BAC) encompassing the entire *Mcpt5* gene. The BAC clone RP23-284A14 from the mouse BAC library RPCI-23 (Osoegawa et al. 2000) was obtained from RZPD (Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany). All modifications were done by homologous recombination in *E. coli* (Zhang et al. 1998) and sequences of all oligonucleotides used for construction by homologous recombination are listed in Table 1. Neighbouring genes contained in the BAC were excluded by replacing several kb of DNA at both ends of the insert by resistance genes (Fig. 1). In a next step, a modified i*Cre* (improved Cre, Shimshek et al. 2002) gene cassette (Testa et al. unpublished) was inserted into the *Mcpt5* gene. The *Cre* cassette was first assembled with a *loxm2*-flanked neomycin-resistance cassette (*neo*) and flanked by homology regions (HR) for recombination into the *Mcpt5* gene. This was done by subcloning the *Cre* cDNA into a minimal vector consisting of the origin of replication and a chloramphenicol resistance gene amplified from pACYC184 (New England Biolabs, Frankfurt, Germany). The primers used for the amplification contain the HRs for both subcloning of *Cre* and insertion into the *Mcpt5* gene (see Table 1). In a next step, the neomycin cassette was inserted 3′ of *Cre* and 5′ of the *Mcpt5* HR. The HR-*Cre*-*Neo*-HR cassette was released from the minimal vector by *Not*I digestion and recombined into the BAC replacing the coding part of exon 1 of the *Mcpt5* gene followed by Cre-mediated deletion of *neo*.

**Table 1** Primers used for transgene construction by homologous recombination

| recombination Step/ Template | Primer | Primer sequence |
|---|---|---|
| shaving of the T7 end of vector backbone<br>Template: pBluescriptIIKS+ | T7HR-Amp-FOR | 5'GCTTGACATTGTAGGACTATATTGCTCTAATAAATTTGCGGCCGCTAATAGGTCTGACCGCTCAGTGGAAC |
| | InsertHR-*Not*I-Amp-REV | 5'TGCCTGTTGGCCTGTGGGAGCCCAGCGAGGTTGGGGCCAGCAGCGGGTGCGCGGCCGCGTGCGCGGAACCCCTATTTG |
| shaving of the SP6 end of vector backbone<br>Template: psiRNA-hH1zeo | InsertHR-*Not*I-Zeo-FOR | 5'GGGCAGAAAGGCTCAGAAGTTGAGACTCAGCAGGGCCTCAGAAAGCTGGTGCGGCCGCCTCCCTTGGAGCCTACCTAGACTCA |
| | SP6HR-Zeo-REV | 5'TTTCTATCCTCCCGAATTGACTAGTGGGTAGGCCTGGCGGCCGCCTGGCCAATGCACTGACCTCCCACATTCCCTT |
| sub-cloning of *Cre*<br>Template: pACYA184 | HR(3'End of Cre)subcloning-HR(last 50bp of *Mcpt5*-Exon1)-*Not*I-minimalVectorFOR | 5'ACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTGTGAGTGCTGGGGTCGCTCCCTCTCCAATCTGCAGGGTCAGCTCCTAAAAGCGGCCGCGCCCTGCACCATTATGTTCCGGA |
| | HR(5'End of Cre)subcloning-HR(50bp upstream of *Mcpt5*-ATG)-*Not*I-minimalVectorREV | 5'ACAGGGAGGGCAGGCAGGTTTTGATGCACAGTCAGCAGGTTGGACACCATATTTGGGCCTTAGGCTTATTACTCCCAGCGGGCAGGGGAGTGGCAGAGCTGCGGCCGCGCCCGGCAGTACCGGCATAACCA |
| insertion of *neo*<br>Template: pPGK-gb2-neo | HR(3'End of Cre)-loxm2-NeoFOR | 5'ACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTATAACTTCGTATATGGTTTCTTATACGAAGTTATCGGCCGCATTCTACCGGGTAGG |
| | HR(last 50bp of *Mcpt5*-Exon1)-loxm2-NeoREV | 5'TTTTAGGAGCTGACCCTGCAGATTGGAGAGGGAGCGACCCCAGCACTCACATAACTTCGTATAAGAAACCATATACGAAGTTATCGCGTTTAAACGGCGCGCCGCACACAA |

Oligonucleotides used for transgene construction by homologous recombination. The respective 50 bp homology regions (HR) were included into the primers used for the amplification of selection markers or the minimal vector

The construct was purified for pronucleus injection as described by Sparwasser et al. (2004) with modifications. Briefly, the construct was separated from the BAC backbone by *Not*I digest and purified by gel electrophoresis and electroelution. Pronucleus injection was done by R. Naumann, MPI of Molecular Cell Biology and Genetics, Dresden, Germany. Genotyping of *Mcpt5-Cre* transgenic mice was performed by PCR (primers: Mcpt5-CreFor 5′ACAGTGGTATTCCCG GGGAGTGT, Mcpt5-CreRev 5′ GTCAGTGCGTT CAAAGGCCA). Founder mice were bred to the ROSA26-EYFP reporter line (Srinivas et al. 2001) and five to 15 week old mice were analyzed for reporter gene expression. Animals were housed under SPF conditions and all experiments were done according to institutional guidelines.

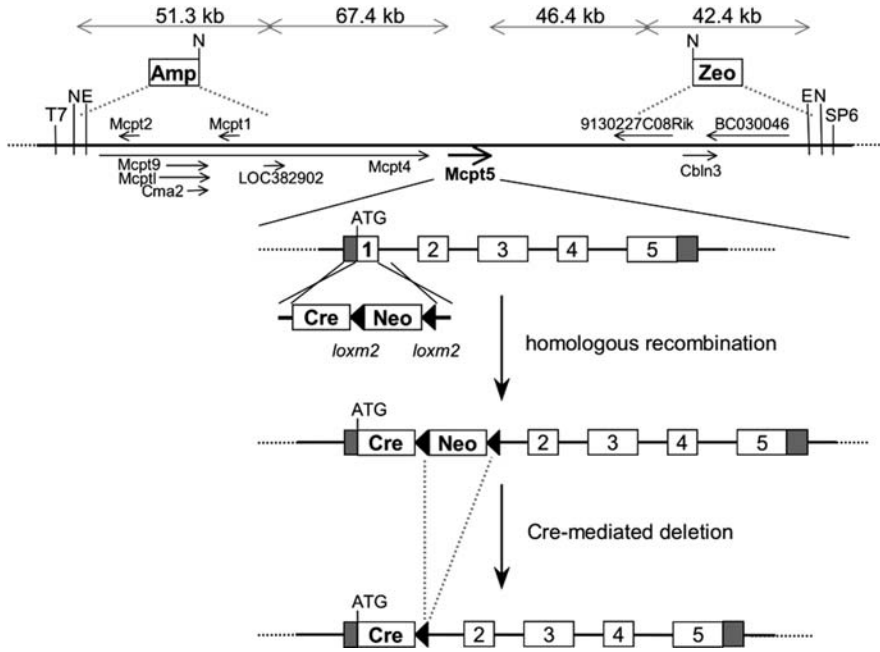


**Fig. 1** Strategy of transgene construction. The BAC RP23-284A14 was shortened by replacing 51.3 kb of insert DNA at the T7 end of the vector backbone and 42.4 kb at the SP6 end by an ampicillin (Amp) and zeocin (Zeo) resistance cassette, respectively, resulting in a residual insert length of 129 kb. A *Not*I restriction site was inserted along with each of the resistance genes. The coding part of the first exon of *Mcpt5* was replaced by a cassette comprising the *Cre* cassette and a *loxm2*-flanked neomycin resistance cassette (Neo) by homologous recombination and subsequent deletion of *neo* by Cre-mediated deletion in *E. coli*. (*Not*I, N, *Eco*RI, I, arrows represent open reading frames, shaded boxes represent untranslated regions)

### Flow cytometric analysis

Total splenocyte suspensions were separated into lymphocyte and non-lymphocyte fractions by magnetic cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) using anti-CD3-Biotin (clone 145-2C11, BD Pharmingen), anti-Biotin MicroBeads, anti-CD19 MicroBeads and LS MACS columns (Miltenyi Biotec). Skin cell suspensions were prepared from shaved abdominal skin plus one ear pinna by mincing followed by an incubation with 2 mg/ml collagenase IV (Worthington Biochem. Corporation, Lakewood, NJ, USA) at 37°C. Cells in peritoneal lavage as well as single cell suspensions from skin and spleen were stained with phycoerythrin (PE)-conjugated anti-CD3 (clone 145-2C11), anti-CD19 (clone 1D3); anti-CD11c (clone HL3), anti-CD45 (clone 30-F11) and anti-Siglec-F (clone E50-2440) (all from BD Pharmingen) or anti-FcεRIα-PE (clone MAR-1), allophycocyanin (APC)-conjugated anti-CD117 (clone 2B8) and anti-CD49b (clone D×5), F4/80-Biotin (clone BM8) and streptavidin-PE (all from eBiosciences, SanDiego, CA) or anti-Gr-1-PE (clone RB6-8C5) (Miltenyi Biotech). The cells were analyzed on a FACSCalibur (BD Biosciences).

## Results

### Transgene construction and screening of transgenic mice for Cre expression

In order to drive Cre expression in mast cells, we used a BAC clone containing the entire *Mcpt5* gene along with abundant upstream and downstream flanking DNA (Fig. 1). Neighboring genes were excluded by shortening the BAC on both ends to avoid possible effects of altered gene dosage in the transgenic mice. In a next step, the coding part of the

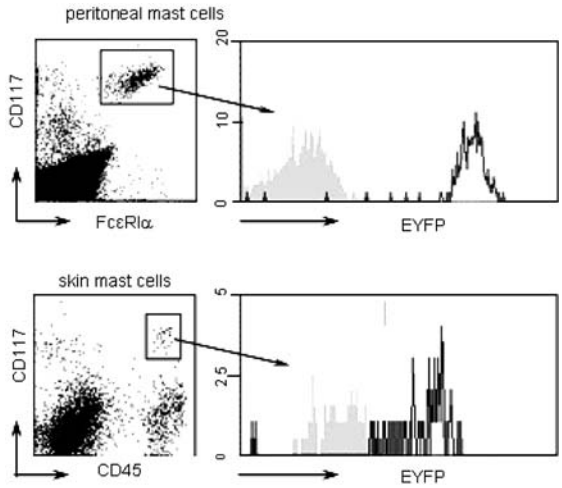


**Fig. 2** Efficiency of Cre-mediated reporter gene activation in mast cells. EYFP expression is demonstrated in cells from peritoneal lavage fluid and single cell suspensions of skin of *Mcpt5-Cre ROSA26-EYFP* double transgenic mice. Peritoneal mast cells were stained for CD117 and FcεRIα (n = 7) and skin mast cells for CD117 and CD45 (n = 4). Mast cell populations were gated and their EYFP fluorescence displayed in histogram plots. The black graph represents *Mcpt5-Cre ROSA26-EYFP* double transgenic mice, the shaded graph represents the *Cre*-negative but *ROSA26-EYFP*-positive control (littermates in most instances)

first exon of *Mcpt5* was replaced by the *Cre* cassette (see Methods and Fig. 1). The construct was released from the BAC backbone by *Not*I digest (Fig. 1) and injected into the pronuclei of C57BL/6 oocytes. Six *Mcpt5-Cre* transgenic founder animals were obtained and mated to a ROSA26 reporter line (Srinivas et al. 2001) which expresses enhanced yellow fluorescent protein (EYFP) under the control of the ubiquitous *ROSA26* promoter following Cre-mediated deletion of a *loxP*-flanked stop element. *Mcpt5-Cre ROSA26-EYFP* double transgenic animals were analyzed for EYFP expression in peritoneal and skin mast cells by FACS as described below (Fig. 2). Reporter gene expression was detected in mast cells from three of the six founder lines. One of these lines showed EYFP expression in only 80% of peritoneal mast cells and was not investigated further. Close to 100% of peritoneal mast cells were EYFP positive in two lines, which, because of autosomal inheritance of the transgene in one line and X-linked inheritance in the other, were called "A-Mcpt5-Cre" and "X-Mcpt5-Cre", respectively.

## Efficient and mast cell-specific Cre-mediated recombination in the lines A-Mcpt5-Cre and X-Mcpt5-Cre

Highly efficient Cre-mediated activation of reporter gene expression was uniformly observed in peritoneal $CD117^+Fc\varepsilon RI\alpha^+$ mast cells of seven *A-Mcpt5-Cre ROSA26-EYFP* double transgenic animals (98.7–99.6%, on average 99.1%, Fig. 2). In skin cell suspensions mast cells were detected in extremely low numbers, on average 0.32%. (Skin mast cells were defined as $CD117^+CD45^+$ cells. A staining of FcεRIα on skin mast cells was not reliable in our hands, likely due to loss of epitopes after enzymatic tissue digestion). In the five animals for which this mast cell population was investigated, the majority of the few $CD117^+CD45^+$ cells gated was EYFP positive (43/47, 54/64, 32/39, 23/27 and 77/88 gated events, Fig. 2). Given that the mast cell gate will inevitably collect also some non-mast cells upon analysis of high total cell numbers (40,000 events), the percentage of skin mast cells expressing EYFP is probably close to 100%. A similar efficiency of Cre-mediated activation of the reporter gene was observed in three male X-Mcpt5-Cre mice (not shown) whereas the two female animals analyzed showed EYFP fluorescence in only 61% and 32% of peritoneal mast cells consistent with random X-chromosome inactivation (not shown).

In both lines, Cre-mediated activation of EYFP expression was specific for mast cells. In seven A-Mcpt5-Cre and three male X-Mcpt5-Cre animals we detected no or only minimal numbers (i.e. below 1%) of EYFP positive cells (below 3% in one A-Mcpt5-Cre animal) in hematopoietic populations other than mast cells (Fig. 3) or in non-hematopoietic cells of the skin cell suspensions (primarily keratinocytes and fibroblasts, not shown). As a control, we used mice with germline deletion of the stop element of the ROSA26-EYFP Cre excision reporter demonstrating that all non-mast cell populations analysed for reporter expression in *Mcpt5-Cre* mice can, in principle, express EYFP (not shown). In contrast to the seven A-Mcpt5-Cre mice (described above) showing mast cell-specific Cre-mediated recombination, one A-Mcpt5-Cre mouse displayed non-specific reporter gene expression in 15–25% of all cell types analyzed including non-hematopoietic cells of the skin (not shown). This finding likely reflects

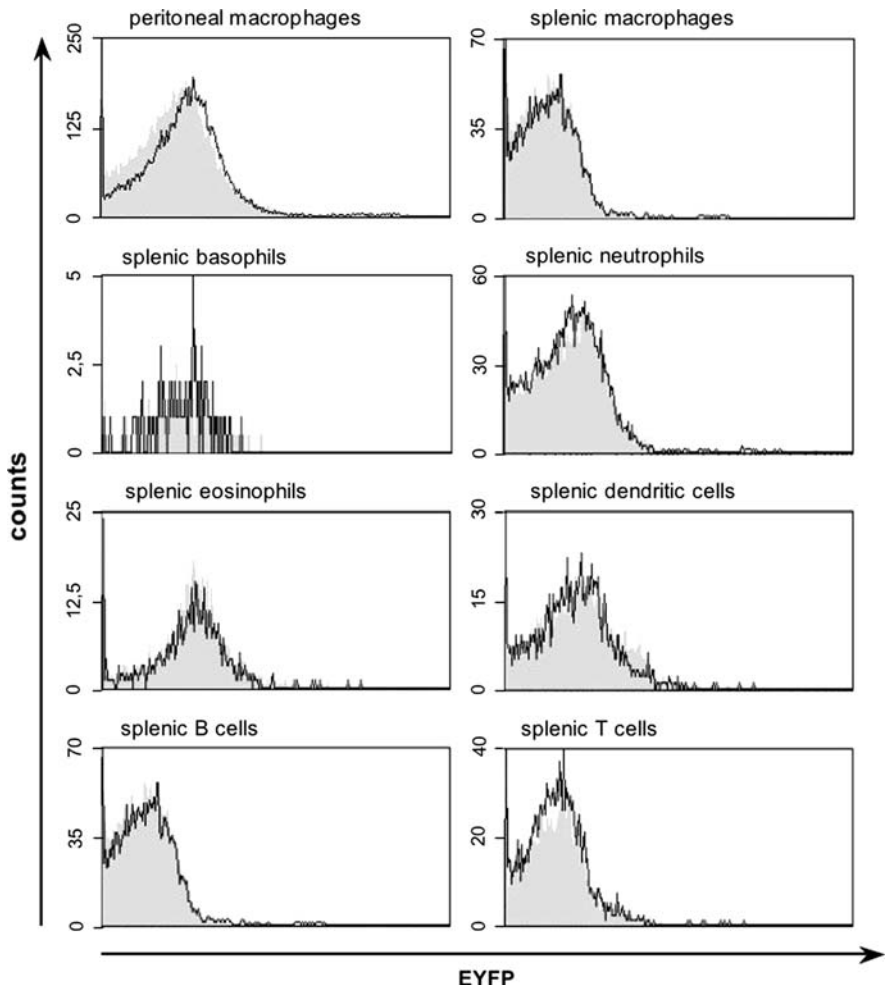


**Fig. 3** Absence of EYFP reporter gene expression in hematopoietic cells other than mast cells. EYFP expression was analyzed in hematopoietic cells from peritoneal lavage and spleen cell suspensions of *Mcpt5-Cre ROSA26-EYFP* double transgenic mice. In order to enrich granulocytes, splenocytes were separated into a lymphocyte and non-lymphocyte fraction by MACS. Basophils were double stained for CD49b and FcεRIα. Macrophages, dendritic cells, neutrophils, eosinophils, B cells and T cells were stained for F4/80, CD11c, Gr-1, Siglec-F, CD19 and CD3ε, respectively. The respective populations were gated and displayed in histogram plots. The black graph represents *Mcpt5-Cre ROSA26-EYFP* double transgenic mice, the shaded graph represents the *Cre*-negative but *ROSA26-EYFP*-positive control (littermates in most instances)

accidental early activity of the transgenic *Mcpt5* promoter.

## Absence of significant Cre-mediated genotoxicity in mast cells of Mcpt5-Cre animals

In order to exclude a reduced proliferative potential of mast cells in *Mcpt5-Cre* transgenic mice due to Cre-mediated genotoxic effects, we compared the percentage of mast cells in peritoneal lavage and the proliferation of peritoneal mast cells from *Mcpt5-Cre ROSA26-EYFP* double transgenic or wild-type mice in vitro. Cell suspensions obtained by peritoneal lavage contained similar numbers of mast cells in wild-type and double transgenic mice (Fig. 4a). Total peritoneal lavage cells were cultured in the presence of stem cell factor. After 23 days, these cultures contained 98% mast cells as judged by expression of CD117 and FcεRIα (not shown). By this time, absolute cell numbers had increased more than 200-fold in both cultures (Fig. 4b). Importantly, the mast cells grown from the *Mcpt5-Cre ROSA26-EYFP* positive mouse uniformly expressed EYFP (not shown) ruling out an overgrowth of the culture by a (hypothetical) minor population of mast cells, which do not express Cre. These results show that *Mcpt5-Cre* positive mast cells are not growth retarded and therefore do not seem to be significantly affected by Cre-mediated genotoxicity.

## Discussion

Here, we describe a novel Cre transgenic mouse allowing mast cell-specific knock out of genes

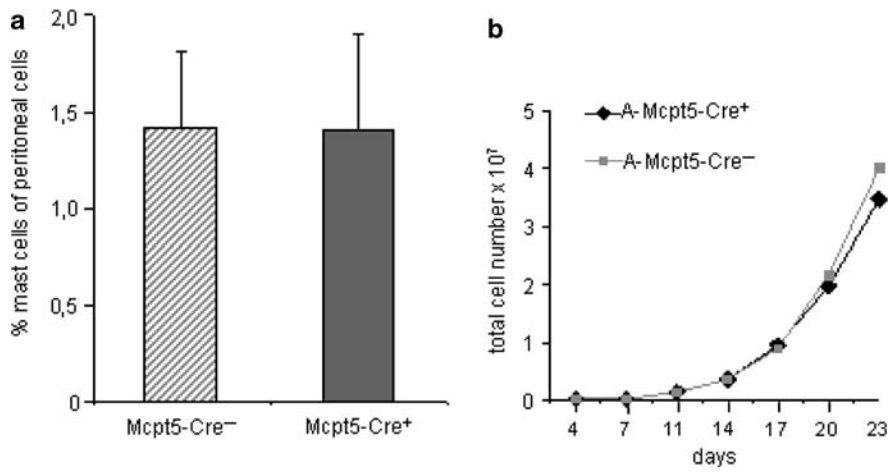


**Fig. 4** Numbers and proliferative potential of peritoneal mast cells in *Mcpt5-Cre* transgenic mice. (**a**) Mast cells were quantified in peritoneal lavage fluid from control (n = 9) and *Mcpt5-Cre* (7 A-Mcpt5-Cre and 3 male X-Mcpt5-Cre) mice. Mast cells were detected as $CD117^{+}Fc\varepsilon RI\alpha^{+}$ cells by FACS. (**b**) Total cells from peritoneal lavage fluid from one control and one *A-Mcpt5Cre ROSA26-EYFP* double transgenic mouse were incubated in complete Opti-MEM supplemented with 4% supernatant from CHO transfectants secreting murine SCF (Malbec et al. 2007). The CHO transfectants were generated by S. Lyman, Immunex, Seattle and were kindly provided by P. Dubreuil. Cell numbers were quantified at each passage every 3–4 days by counting trypan blue-excluding cells

in vivo. We employed BAC transgene technology to express a *Cre* cassette under the control of the *Mcpt5* promoter. To our knowledge, this is the first report of mast cell-specific Cre-mediated recombination in the mouse.

To date, important insights into functions of mast cells have been gained by reconstitution of mast cell-deficient mice with mast cells differentiated in vitro from gene deficient bone marrow or embryonic and hematopoietic stem cells (Galli et al. 2005b; Grimbaldeston et al. 2005; Kitamura et al. 1978; Tsai et al. 2005). While this model yielded important information, inherent technical problems limit experimentation and interpretation of data in this system (Tsai et al. 2005). We expect that mast cell-specific knock out of genes using our new *Cre* transgenic line will greatly facilitate investigation of mast cell biology.

The two *Cre* transgenic lines demonstrate that the *Mcpt5*-promoter allows efficient and cell type-specific expression of a *Cre* transgene inserted into the first exon. One animal of the line A-Mcpt5-Cre (characterized by autosomal transgene inheritance) showed non-specific Cre-mediated recombination in non-mast cells while seven animals of this line did not. This finding likely reflects accidental early activity of the transgenic *Mcpt5* promoter, e.g. in one stem cell during the embryonic four cell stage. Future analysis will define the frequency of such events more precisely. In females of the X-Mcpt5-Cre line (X-chromosomal inheritance), Cre-mediated recombination occurred in only about half of the mast cells due to inactivation of the transgene-carrying X-chromosome. Incomplete inactivation of particular genes may be advantageous if the attenuation of a severe phenotype is desired.

Cre recombinase has been shown to mediate genotoxicity in some *Cre* transgenic or *Cre* knock in mouse strains resulting in a reduced proliferative potential or even death of Cre expressing cells (Schmidt-Supprian and Rajewsky 2007). In *Mcpt5-Cre* mice, however, we found no difference in mast cell numbers or proliferation of mast cells *ex vivo* in response to stem cell factor indicating that Cre-mediated genome damage is not a prominent feature of these mice.

We are currently refining our system further to achieve also inducible mast cell-specific gene inactivation in adult mice. In addition, we are breeding the new lines to iDTR mice (Buch et al. 2005) aiming at diphtheria toxin-inducible ablation of the mast cell lineage in adult animals.

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