Tetracycline-controlled transgene activation using the ROSA26-iM2-GFP knock-in mouse strain permits GFP monitoring of DOX-regulated transgene-expression

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Abstract

Background: Conditional gene activation is an efficient strategy for studying gene function in genetically modified animals. Among the presently available gene switches, the tetracycline-regulated system has attracted considerable interest because of its unique potential for reversible and adjustable gene regulation.

Results: To investigate whether the ubiquitously expressed Gt(ROSA)26Sor locus enables uniform DOX-controlled gene expression, we inserted the improved tetracycline-regulated transcription activator iM2 together with an iM2 dependent GFP gene into the Gt(ROSA)26Sor locus, using gene targeting to generate ROSA26-iM2-GFP (R26t1Δ) mice. Despite the presence of ROSA26 promoter driven iM2, R26t1Δ mice showed very sparse DOX-activated expression of different iM2-responsive reporter genes in the brain, mosaic expression in peripheral tissues and more prominent expression in erythroid, myeloid and lymphoid lineages, in hematopoietic stem and progenitor cells and in olfactory neurons.

Conclusions: The finding that gene regulation by the DOX-activated transcriptional factor iM2 in the Gt(ROSA) 26Sor locus has its limitations is of importance for future experimental strategies involving transgene activation from the endogenous ROSA26 promoter. Furthermore, our ROSA26-iM2 knock-in mouse model (R26t1Δ) represents a useful tool for implementing gene function in vivo especially under circumstances requiring the side-by-side comparison of gene manipulated and wild type cells. Since the ROSA26-iM2 mouse allows mosaic gene activation in peripheral tissues and haematopoietic cells, this model will be very useful for uncovering previously unknown or unsuspected phenotypes.

Background

To understand the detailed function of particular genes we must be able to translate analytical and database information into experimental model systems. In this respect, the laboratory mouse can be used as a higher vertebrate model that permits the application of genetic tools for the in vivo analysis of gene function. Since constitutive, global expression of transgenes is often associated with embryonic lethality or unwanted pleiotropic effects, tight postnatal transgene regulation is the method of choice. Furthermore, correlations between phenotypes and ‘ON’ and ‘OFF’ states of the regulated gene can provide reliable cues for understanding gene-function. However, to ensure that the observed phenotypes are correlated with the gene function under investigation the reservoir of genetic tools has to be carefully evaluated.

Over the last decade the tetracycline (tet)-regulatory system has been extremely useful for generating conditional transgenic mouse models (for reviews see [1-3]). Besides its fundamental appeal as an established reversible regulatory system in mice, tet ‘ON/OFF’-systems...
have attracted considerable interest because of their unique potential for reversible gene regulation, with the additional advantage of allowing adjustment of transgene expression levels through the administration of predetermined amounts of the pharmacological inducer doxycycline (DOX) [4-6]. The graded response of tet-regulated transgenes thus provides the unique opportunity to analyze gene dosage effects in living animals.

Although a large collection of tet-controlled transgenic mice suitable for studying gene function in many different cell types is available to the scientific community (http://www.zmg.uni-mainz.de/tetmouse for an electronically searchable database), no comprehensive analysis of a tet-regulatory effector line using the ubiquitously expressed Gt(Rosa)26Sor gene locus has been published. Therefore, we generated a tet ‘ON’ knock-in mouse line (R26Δ1A) containing the iM2 reverse DOX-dependent transactivator coding region [7] and an iM2 responsive GFP gene at the Gt(Rosa)26Sor gene locus. Analysis of R26Δ1 mice using different iM2-responsive reporter strains revealed mosaic induction of conditional reporter gene activity in peripheral organs and in hematopoietic cells. No substantial iM2 reporter gene activation was detected in most parts of the adult brain with the exception of the bulbus olfactorius and, to a lesser extent, the striatum.

Results

Generation of the ROSA26-iM2PtetGFP knock-in mouse

By gene targeting, we inserted the iM2 transactivator gene [7], a loxP flanked neo/ura selection marker and a Ptet-controlled GFP followed by a human growth hormone polyadenylation signal into the XbaI site between exon1 and 2 of the Gt(Rosa)26Sor locus (Figure 1A). A splice acceptor sequence (SA) identical to the one used in the original lacZ-gene-trap allele [9] was inserted 5′-prime to the iM2 start codon to facilitate the processing of ROSA26 promoter controlled transcripts [10]. A synthetic intron in the 5′UTR of GFP was used to stabilize the Ptet transcript and an additional human growth hormone polyadenylation sequence was inserted in front of Ptet to avoid transcriptional interference between the ROSA26 and Ptet promoter. The recombiant gene locus Gt(Rosa)26t1 (R26Δ1) (Figure 1A) is thus expected to express the iM2 transactivator from the endogenous ROSA26 promoter. In the presence of DOX, iM2 can activate the Ptet-promoter, which in turn can be visualized by the expression of GFP. Thus, colocalisation of both iM2 and the Ptet responder GFP genes in the same locus facilitates a rapid fluorescent screen for monitoring the Ptet-activity in single cells.

The generation of the R26Δ1 gene locus by homologous recombination in the genome of ES cells was pre-screened by PCR and confirmed by Southern blotting (Figure 1B). Six independent ES cell clones with insertion of the inducible iM2-PtetGFP module at the Gt (Rosa)26Sor locus were identified (Figure 1B). All six ES cell clones showed DOX-inducible GFP fluorescence (Figure 1B) and ES cell clone 64 was used for blastocyst injections to generate the ROSA26-iM2-PtetGFP mouse line, designated R26Δ1. Removal of the loxP flanked neo/ura selection cassette was performed by in vivo excision using the deleter-cre strain [11] thus generating the R26Δ1Δ allele in a sub-line (R26Δ1Δ) lacking the neo/ura genes (Figure 1C).

In embryos, spleen, kidney and liver of R26Δ1Δ mice rapid amplification of cDNA ends (RACE) revealed that R26Δ1Δ transcripts contained 3′-exon1 sequences of ROSA26 transcript variant 2 (Figure 1C). Since the ROSA26 promoter and exon1 of splice variant 2 were not present in the targeting vector, this finding confirms that the recombined R26Δ1Δ gene locus drives iM2 expression from the ROSA26 promoter. Thus the presence of splice variant exon1 at the 5′-end of iM2 transcripts provides additional proof for successful iM2 gene module insertion into the Gt(Rosa)26Sor gene locus. Currently, we cannot resolve why the exon1 splice variant 1 was not detected in our analysis. Because sequence alteration at the exon1 splice donor site could be excluded by sequencing it is possible that the presence of a second artificial intron in front of the iM2 coding sequence might influence the maturation of the primary R26Δ1Δ transcript.

The presence of the neo spacer sequence and the gene dosage effect on the DOX regulated GFP in the ROSA26-iM2-GFP locus

The iM2 activity in R26Δ1Δ and R26Δ1Δ/Δ1Δ mice was determined by GFP-detecting immunoblots of total protein extracts obtained from different tissues (Figure 2). Calnexin was used as loading control and found to be differentially expressed in various tissues. As shown in Figure 2, in the absence of DOX no GFP-specific signal was detected in any tissue of all three genotypes as exemplified for muscle tissue of R26Δ1Δ and R26Δ1Δ/Δ1Δ mice. This directly indicates that the Ptet promoter is not activated after insertion into the Gt(Rosa)26Sor locus by read through transcripts or promoter interference. When mice were exposed to DOX, we detected weak GFP signals only in thymus extracts from R26Δ1Δ mice (not shown). However, in R26Δ1Δ mice highest GFP levels were obtained following DOX induction in spleen, thymus, muscle and testis (Figure 2). Thus - as noticed earlier and described in detail for the Gt(Rosa)26Sor gene locus in ES cells [12] - removal of the NEO gene improved gene expression at the targeted locus. In homozygous R26Δ1Δ/Δ1Δ mice GFP expression was even further augmented in muscle and testis but this increase
The gene targeted R26t1 allele controls the expression of DOX-inducible transcription from the endogenous promoter of the Gt(ROSA)26Sor locus. A) Gt(ROSA)26Sor and Gt(ROSA)26t1 (R26Rt1) loci. Exons are indicated as grey boxes; light grey show alternative spliced exons. Genetic elements inserted at the first XbaI site in intron1 of Gt(ROSA)26Sor are given in coloured boxes: first (yellow), the adenovirus major late splice acceptor sequence followed by the small intron of the adenoviral tripartite leader and the iM2 coding region terminated by the human growth hormone polyadenylation signal (hgh_pA); second (red), a loxP site (black triangles) flanked neomycin/uracil selection cassette [36] terminated by hgh_pA; third (green), the PtetO5-GFP gene module terminated by SV40_pA. Diagnostic restriction sites are indicated (E = EcoRV, Xh = XhoI, X = XbaI, P = PacI, A = AscI). The transparent green bar represents the chromosomal region covered by the targeting vector. Red-framed black boxes below R26t1 mark positions of Southern blot probes that detect restriction fragments given as red lines in kilo bases (kb).

B) Left: Southern blots of genomic DNA isolated from wild type (WT) and PCR-preselected ES cell clones 27, 48, 60, 64, 65 and 67. Right: Images of R26t1 ES cells show DOX-dependent GFP expression.

C) Schematic representation of the R26t1Δ allele after Cre mediated removal of neo/ura at the R26t1 allele (symbols are as in 1A). Transcriptional starts of the ROSA26 and Ptet promoter are indicated by arrows. Below the 5'-RACE identified exons (solid lines) and introns (dashed lines) of the iM2 encoding R26t1Δ mRNA are given. Below the 5' ends of mRNA from embryos, kidney liver and spleen are aligned to Gt(ROSA26Sor) transcript variants of the database (NCBI Accession numbers are indicated).
Figure 2: Expression of the GFP reporter gene in adult R26t1Δ and homozygous R26t1Δ/t1Δ mice. Western blot analysis of muscle extracts from wild-type (WT), R26t1Δ, and R26t1Δ/t1Δ mice three to five month of age. Upper part: No GFP-specific signal could be detected in muscle without DOX administration. The very same blot was probed for calnexin (Cal.) and the result is shown below the GFP-specific signal. Lower part: Western blot analysis of GFP (four upper lanes) and calnexin (four lower lanes) expression in spleen, thymus, muscle and testis extracts from DOX-induced wild-type (WT), heterozygous (R26t1Δ) and homozygous (R26t1Δ/t1Δ) mice. The GFP and calnexin signals were obtained from the same blot. For reasons of simplicity the calnexin loading control and the GFP signal are aligned and pictured as one group. Animals were treated with DOX for 3 weeks prior analysis.
was not obvious in spleen and thymus. However, since spleen and thymus represent tissues with high GFP expression in activated R26G1A animals, a two-fold increase in R26G1A mice might have escaped our semi-quantitative analysis. Thus it seems that for tissues with low levels of iM2 controlled genes a gene dosage increase of iM2 and the Ptet gene(s) or both can be beneficial for the expression level of Ptet controlled genes.

**Characterisation of the R26G1A effector mouse in hematopoietic tissues**

To determine to what extend different hematopoietic cell types of R26G1A mice can implement DOX-regulated transgene expression, DOX-induced and non-induced R26G1A mice were analyzed by flow cytometry. As expected, no GFP-expressing hematopoietic cells were found in R26G1A mice never exposed to DOX (data not shown). In DOX-treated R26G1A littermates GFP-expressing cells were present in all analyzed hematopoietic lineages (Figure 3). Based on their expression for CD71 and Tert19 four subpopulations representing different red blood cell maturation steps could be established (erythrocytes, maturation stage I to IV). Compared to the immature early proerythroblasts (I, 11.01% ± 6.4), GFP+ cells were less abundant in more mature erythroid populations (II-IV). Analysis of bone marrow granulocytes demonstrated that about one fourth (24.15% ± 8.37) of the less mature CD11b+/Gr1low population expressed GFP whereas the more differentiated CD11b+/Gr1high population contained only few GFP-expressing cells (2.05% ± 1.43). In the spleen of DOX-induced R26G1A mice 10.64% ± 7.16 of the granulocytes were GFP positive (CD11b+/Gr1+ splenic granulocytes). Within the megakaryocytic lineage CD41+/c-Kit+ megakaryocytic progenitors contained 19.69% ± 13.07 GFP-expressing cells while within the CD41+/c-Kit− population of more mature megakaryocytes only 3.39% ± 3.9 of cells expressed GFP. Similarly, flow cytometric analysis of CD23+ mast cells revealed the presence of a fraction of GFP-expressing cells (11.58% ± 5.94). GFP+ cells were also present in the lineage negative (lin−), c-Kit- and Sca-1-expressing population of cells that contains hematopoietic stem cells and progenitors (12.21% ± 7.68, LKS stem cells). From the analyzed B-lymphocytes a high percentage of B220+/CD19− pre-pro B cells (42.6% ± 11.54), re-circulating B220+/CD19low (25.48% ± 17.04) and B220+/CD19high new produced (21.41% ± 11.05) B-cells expressed the GFP reporter gene. Within the thymus GFP-expressing cells were found in CD4+/CD8− double positive cells (11.51% ± 6.32) and also in CD4 single positive (6.01% ± 2.88) and CD8 single positive (12.14% ± 6.38) thymocytes. These results indicate that the expression of the R26G1A controlled iM2 transactivator can activate the co-localized Ptet-GFP transgene in erythroid, myeloid, lymphoid lineages and also in lin−/c-Kit+/Sca-1+ hematopoietic stem and progenitor cells in presence of DOX. Our findings further demonstrate that the percentages of Ptet-GFP reporter gene expressing cells are highly variable in different hematopoietic cell types of R26G1A.

**Evaluation of the R26G1A effector mouse in the brain**

Several Gt(ROSA)26Sor targeted Cre-reporter mouse lines are available and have been successfully used to monitor Cre-induced transgene activation patterns in different cell types of the mouse brain [8,10,13-15]. However, results obtained with the endogenous ROSA26 promoter used for conditional gene expression in the brain were not consistent [16-19]. To clarify the potential of the R26G1A mice for conditional transgene expression in neural tissues, protein extracts from different brain areas of R26G1A and R26G1A mice were analyzed by Western blotting (Figure 4A). A very strong DOX-dependent GFP-specific signal was present in protein extracts from the olfactory bulb of R26G1A mice. In contrast, no GFP signal was detected in any other brain region after DOX-induction as shown for the mesencephalon, the hippocampus, the cortex and the cerebellum (Figure 4A). In homozygous, DOX-induced R26G1A mice weak GFP expression was found in cerebellar extracts and to an even lower extent in the mesencephalon, the hippocampus and the cortex. The GFP signal in the olfactory bulb was dramatically increased and ten fold less protein was loaded for immunoblotting to obtain a comparable GFP signal for the calnexin loading control.

Immunohistochemistry of sagittal brain sections confirmed strong, DOX-dependent GFP-specific expression in the olfactory bulb of R26G1A and R26G1A mice (Figure 4B). However, the GFP-staining was restricted to the glomeruli formed between axonal projections of olfactory receptor neurons (ORNs) and dendritic processes from mitral cells of the olfactory bulb. Since the cell bodies of mitral cells within the olfactory bulb were negative for GFP immunostaining, the GFP signal in the olfactory bulb is derived from ORNs, which have their cell bodies in the nasal cavity of the olfactory epithelium outside the brain. Hardly any GFP-positive cells were apparent in brain sections of DOX-induced R26G1A and R26G1A mice; with the exception of sparse GFP-positive cells in the striatum and hypothalamus of homozygous R26G1A mice (Figure 4B) directly confirming the immunoblot results (Figure 4A). Thus, as noticed in peripheral tissues, the increased gene dosage of the R26G1A allele improved the expression of GFP in the brain. Our findings therefore suggest that in heterozygous R26G1A mice, a critical threshold of DOX-activated
Figure 3 The R26<sup>rt1</sup> mouse line directs transgene expression to adult blood cells and hematopoietic stem and progenitor cells.

(A) Expression of GFP in different hematopoietic cells is given as mean values ± standard deviations in a bar graph. Analyzed cell-lineages are shown on the right. For conditional GFP activation three month old mice were exposed to DOX for fourteen days prior to the analysis. No GFP activation was detected in genetically identical littermates not exposed to DOX (not shown). At least four different animals from DOX-induced and non-induced littermate controls were analyzed for hematopoietic lineage determination.

(B) In the right column relative percentages of GFP<sup>+</sup> cells are indicated as mean values ± standard deviations. Lineage-specific markers are specified in the central white column. Roman numerals refer to different maturation stages of red blood cell development: I, proerythroblasts; II, basophilic erythroblasts; III, late basophilic erythroblasts and chromatophilic erythroblasts; IV, orthochromatophilic erythroblasts. GFP, green fluorescent protein; LKS, lineage-/c-Kit<sup>+</sup>/Sca1<sup>+</sup>.

| LINEAGE                                      | MARKERS                      | GFP<sup>+</sup> Cells (relative %) ± s.d. |
|----------------------------------------------|-------------------------------|----------------------------------------|
| erythrocytes (maturation stage I)            | CD71<sup>+</sup>/TER119<sup>+</sup> | 11.01±6.40                             |
| erythrocytes (maturation stage II)           | CD71<sup>+</sup>/TER119<sup>+</sup> | 1.01±1.00                              |
| erythrocytes (maturation stage III)          | CD71<sup>low</sup>/TER119<sup>+</sup> | 1.12±1.53                              |
| erythrocytes (maturation stage IV)           | CD71<sup>+</sup>/TER119<sup>+</sup> | 0.58±0.60                              |
| immature granulocytes                        | CD11b<sup>+</sup>/Gr-1<sup>low</sup> | 24.15±8.37                             |
| mature granulocytes                          | CD11b<sup>+</sup>/Gr-1<sup>high</sup> | 2.05±1.43                              |
| splenic granulocytes                         | CD11b<sup>+</sup>/Gr-1<sup>+</sup> | 10.64±7.16                             |
| megakaryocytic progenitors                   | c-Kit<sup>+</sup>/CD41<sup>+</sup> | 19.69±13.07                            |
| mature megakaryocytes                        | c-Kit<sup>+</sup>/CD41<sup>+</sup> | 3.39±3.90                              |
| mast cells                                   | CD23<sup>+</sup>              | 11.58±5.94                             |
| LKS                                          | lineage<sup>-</sup>/c-Kit<sup>+</sup>/Sca1<sup>-</sup> | 12.21±7.68                             |
| pre-pro B-cells                              | B220<sup>+</sup>/CD19<sup>+</sup> | 42.60±11.54                            |
| re-circulating B-cells                       | B220<sup>+</sup>/CD19<sup>low</sup> | 25.48±17.04                            |
| new produced B-cells                         | B220<sup>high</sup>/CD19<sup>high</sup> | 21.41±11.05                            |
| double positive T-cells                      | CD4<sup>+</sup>/CD8<sup>+</sup> | 11.51±6.32                             |
| helper T-cells                               | CD4<sup>+</sup>              | 6.01±2.88                              |
| cytotoxic T-cells                            | CD8<sup>+</sup>              | 12.14±6.32                             |

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http://www.biomedcentral.com/1471-213X/10/95
iM2 for transcriptional activation of Ptet-GFP is not reached in most cells of the brain.

In order to increase the level of active transactivator in the brain, we generated R26^{tA} mice that express the DOX-activated transcription factor tTA in the forebrain by breeding R26^{tA} and TgCamKII-tTA mice [20]. Compared to R26^{tA} and R26^{tA/tA} mice, adult R26^{tA/tA}/TgCamKII-tTA animals showed many more GFP-positive cells in brain regions with strong TgCamKII-tTA expression including striatum, cortex and glomeruli of the olfactory bulb thus favouring the hypothesis that detectable brain-specific GFP expression needs a certain threshold of DOX-induced activator molecules (Figure 4C). However, only a few pyramidal cells in hippocampal layer CA1 demonstrated GFP expression (Figure 4D) despite the high activity of tTA in those brain regions [20,21]. This lack of optimal tTA mediated Ptet-GFP gene activation in the R26^{tA} locus might be caused by a negative interference of the CamKII promoter expressed tTA and R26^{tA} encoded iM2 forming inactive heterodimers. The formation of inactive heterodimers is supported by mice expressing iM2 and tTA together with a Ptet-GFP responder transgene TgPtetbi-GFP-GluA1/nlacZ [21]. As described by Mack et al., TgCamKII-tTA/TgPtetbi-GFP-GluA1/nlacZ mice show high numbers of GFP positive cells in the forebrain
However, in littermate animals in addition harbouring the R26\textsuperscript{Δ}; the GFP signal dramatically dropped (Figure 4D, right) demonstrating reduced activity of the tTA transactivator in presence of the R26\textsuperscript{Δ} allele. In summary, our results indicate that a certain threshold level of iM2 expression is needed to activate Ptet promoters and provide direct evidence that the observed lack of Ptet-mediated GFP activation in DOX induced R26\textsuperscript{Δ} mice might not just be the result of poor penetration of DOX through the blood brain barrier.

**Activation of R26\textsuperscript{Δ} controlled expression of Ptet regulated transgenes is DOX-dependent and inhomogeneous**

To further evaluate the functional capability of iM2 in R26\textsuperscript{Δ} mice, we recruited two different Ptet-transgenic mouse lines. First, a transgenic mouse line co-expressing Ptet-controlled Wnt1 and luciferase (TgPtet-Wnt1-IRES-luciferase) [22] to detect very low levels of transgene induction by non-invasive bioluminescence imaging [23]; second, a Ptetbi-controlled GFP/lacZ line for cellular resolution of iM2 activity (TgPtetbi-GFP/lacZ) (Figure 5).

Compound R26\textsuperscript{Δ}/TgPtet-Wnt1-IRES-luciferase mice were either treated with DOX in the drinking water or were never exposed to DOX and after luciferin injection bioluminescence levels were recorded (Figure 5A).

DOX-exposure during three days induced significant levels of luminescence whereas no bioluminescence signal was monitored in mice never exposed to DOX. Consistent with the previously reported variable levels of transgene expression from the Gt(ROSA)26Sor locus in different adult mouse tissues [16,17,24], the luminescence was not uniformly distributed in DOX-induced R26\textsuperscript{Δ}/TgPtet-Wnt1-IRES-luciferase mice. This inhomogeneous luciferase activity pattern thus confirms our immunoblot observation that the R26\textsuperscript{Δ} locus leads to highly variable Ptet-transgene expression.

Next, R26\textsuperscript{Δ} induced Ptet-gene activation was visualized on a cellular level in compound R26\textsuperscript{Δ}/TgPtetbi-GFP/lacZ mice by X-Gal staining (Figure 5B). As expected, no β-galactosidase (β-gal) expressing cells were detected without DOX treatment (data not shown). In line with the DOX-dependent induction of luciferase expression (Figure 5A), compound R26\textsuperscript{Δ}/TgPtetbi-GFP/lacZ animals treated for two weeks with DOX showed lacZ reporter gene activity in skin, tongue, colon, small intestine, pancreas, lung, kidney, testis and liver. Interestingly, β-gal expression in these organs was restricted to subsets of cells. No β-gal\textsuperscript{+} cells were detected in heart, bladder and stomach (Figure 5B).

Taken together our results indicated that the expression of conditional Ptet transgenes can be tightly regulated with the R26\textsuperscript{Δ} allele and that the R26\textsuperscript{Δ} mouse
model implements mosaic Ptet-transgene activation in a number of peripheral tissues.

Discussion
The promoter of the $Gt(ROSA)26Sor$ locus is frequently used for ubiquitous expression of reporter genes in the mouse. These animal models represent valuable tools for conducting conditional gene expression studies. However, it is debated whether the endogenous promoter of the $Gt(ROSA)26Sor$ locus can direct conditional transgene activation to all tissues of adult mice [16-19].

Now we provide additional information by a qualitative and quantitative analysis of a gene targeted mouse line (R261Δ) that contains the DOX-inducible iM2 transactivator together with an iM2-dependent GFP gene inserted into the $Gt(ROSA)26Sor$ locus. We could show that after gene targeting the ROSA26 promoter drives expression of the inserted transcriptional activator gene in several tissues of R261Δ mice. In embryo, liver, spleen and kidney exon1 of the ROSA26 transcript variant 2 was used in iM2 transcripts. If and to what extent exon1 of transcript variant 1 is used in iM2 transcripts cannot be concluded from our data, since the conducted RACE analysis did not provide a quantitative analysis of all transcripts of the ROSA26 promoter in R261Δ mice. More importantly, exon1 transcript variant 2 is located outside of our targeting vector providing additional evidence that the $Gt(ROSA)26Sor$ targeting was successful. Since electroporation with our targeting vector provided several randomly inserted GFP expressing ES cell clones, all containing transcriptional start sites in the short arm of the targeting vector (data not shown), the transcript analysis conducted here provides direct experimental proof for the ROSA26 promoter-specific transcription of the iM2 transgene and can be used as a functional test for demonstrating correct gene transcription of the rearranged locus.

In gene targeted mice ROSA26 promoter controlled iM2 activity was evaluated by monitoring (i) the co-inserted downstream Ptet-GFP reporter gene, (ii) the conditional induction of the TgPtet-Wnt1-IRES-luciferase [22] or the TgPtetbi-GFP/IacZ [25] transgenes. All three different reporter systems, including the very sensitive luciferase reporter, demonstrated convincingly that the R261Δ allele permits gene induction with no background activity of the iM2-controlled gene in the non-induced state. These results indicate that illegitimate activation of Ptet-GFP in the $Gt(ROSA)26Sor$ locus by read through transcripts or promoter interference does not take place. Our experiments also demonstrate that different iM2 responder genes could be induced by DOX. However, the responder gene expression was moderate and highly variable between and within different tissues. We detected conditional transgene expression in a subset of cells from skin, tongue, colon, small intestine, pancreas, lung, kidney, testis and liver, whereas some organs like heart, bladder and stomach had no detectable transgene induction.

In addition to the localization of conditionally activated cells within peripheral organs, we provide a detailed picture of R261Δ encoded iM2 expression in the hematopoietic system. Although for the initially gene-trapped β-Geo ROSA26 mouse strain the expression of the integrated β-Geo reporter gene in immature red blood cells, lymphoid and myeloid lineages was found [24] and several ROSA26-driven reporter mouse strains exist and have been used in hematopoietic tissues [8,10,13-15], no detailed information about the potential and tissue-specificity of ROSA26-driven tet ‘ON/OFF’ mouse systems is available for hematopoietic tissues. To provide this missing information, we analyzed induced R261Δ mice by flow cytometry. The results of these experiments indicate that the R261Δ allele is active in different adult blood cell types and also in the lineage negative c-Kit- and Sca-1-expressing (LKS) population, which contains hematopoietic stem cells and progenitors.

In contrast to the peripheral tissues and the hematopoietic system, in cells of the central nervous system the activity of the R261Δ encoded iM2 was barely detectable. Olfactory receptor neurons projecting to glomeruli of the olfactory bulb showed the highest DOX-inducible iM2 activity in homozygous R261Δ/Δ and heterozygous R261Δ mice. In whole brain extracts, however, only low GFP protein levels could be detected in mesencephalon, hippocampus and cortex of DOX-induced homozygous R261Δ/Δ mice. Immunohistochemical staining of brain sections revealed a few, scattered GFP-expressing cells. The low levels of functional iM2 appeared to be one reason for the dysfunction of the R261Δ allele in most cells of the central nervous system since an increase of the R261Δ gene dosage in homozygous mice provided higher GFP expression levels in several brain regions including the glomeruli of the olfactory bulb. Even more neurons showed R261Δ-derived GFP expression when the levels of functional transactivator were increased by forebrain specific tTA expression using a CaMKII promoter driven transgene [20]. However, GFP expression was lower than in other transgenic Ptet-GFP responder mice, most likely due to the presence of the iM2 in R261Δ animals. The reduced tTA activity in R261Δ genotypes therefore is best explained by the mutual interference of tTA and rtTA (iM2) heterodimers [26,27] and can be convincingly visualized by the reduction of GFP-GluA1 expression in the CA1 pyramidal neurons of the hippocampus in compound transgenic mice.

Applying a very similar targeting strategy, Bäckman and colleagues recently generated a ROSA26-rtTA
knock-in mouse containing a Ptet-Cre responder element inserted downstream of a ROSA26-driven rtTA cassette. Interestingly, DOX-induced adult mice expressed low Cre mRNA levels and therefore failed to activate recombination of a floxed reporter gene suggesting that the endogenous ROSA26 promoter might be too weak for efficiently inducing conditional transgene activation [28]. Besides the low activity of the endogenous ROSA26 promoter, a second reason for the poor iM2 activity in the brain might be the blood brain barrier, which may limit the free accessibility of DOX for neurons in the brain. However, using intra-cerebral DOX injection or rAAV virus mediated tTA gene delivery into the brains of adult R26\textsuperscript{1A} mice, we failed to achieve neuronal GFP expression in the injected cortex or hippocampal areas (data not shown). Thus as described in previous studies [7] the Ptet promoter might be subject to epigenetic gene silencing when not activated during early stages of development. In this respect it is of note that in all experiments studying the expression of the R26\textsuperscript{1A} GFP allele in the brain, we already applied DOX to the embryo. Similarly, we used tTA expression to activate the R26\textsuperscript{1A} encoded GFP in the forebrain since the CamKII promoter of Tg-CamKII-tTA is transcriptionally active in the mouse brain during early development [25,29] and thus the Ptet promoter region is still open for transcriptional activation.

Currently, we can not formally exclude the possibility that, in addition to other effects, the lack of detectable Ptet activation in the brain might in part be caused by transcriptional interference, which is known to reduce or extinguish transcriptional activity of downstream promoters in double gene constructs [30-32] and was recently described for the CMV and the ROSA26 promoter in a targeted Gt(Rosa)26Sor locus in ES cells [12]. However, the fact that the R26\textsuperscript{1A}-iM2 activation of transgenic Ptet-responders in trans was limited as well, and that in the SK3 channel the insertion of a very similar linked transactivator and responder gene was operative [33] strongly argues against promoter interference. In addition, negative expression variation effects of Ptet caused by its 91 bp fragment from the CMV promoter can be responsible for the inhomogeneous activity of the Ptet promoter.

**Conclusion**

The presented qualitative and semi-quantitative analysis of our R26\textsuperscript{1A} mouse line provides detailed data on the iM2 and Ptet controlled GFP activity, which is a key information for future experiments using R26\textsuperscript{1A} mice. This knowledge can be useful to guide the experimental design of particular research projects using DOX-regulated gene expression. Finally, the here conducted analysis provides valuable information about the potential of the R26\textsuperscript{1A} mouse for activating transgene expression in different tissues and hematopoietic lineages and thus will help to decide if this mouse model is suitable for a particular *in vivo* experiment.

**Methods**

**Construction of the targeting vector**

For gene targeting at the Gt(Rosa)26Sor locus we inserted the coding sequence for the iM2 transactivator (rtTA-M2; [7]), containing a codon improved version of the tet-inducible M2 transcription factor [34], a loxP flanked neo selection marker and a Ptet-controlled GFP followed by a high polyadenylation signal into the Pac1/Ascl site between exon1 and 2 targeting vector pROSA26PA [8] to generate plasmid pROSA-iM2. In detail: a plasmid containing the tri-TAUBi-AF cassette was used as starting material [35,36]. The unique KpnI and ClaI sites were removed from phM2-1 by filling in with Klenow polymerase followed by ligation. A 120 bp fragment containing the adenovirus major late transcription splice acceptor sequence (SA) from the intron1/exon2 boundary was amplified by PCR from pSAbGal [9] using primer SA-F (5’-TTTGGCCCATACTGCTTAATTAATGGGGCGCACTATGTCAGGG-3’) and SA-R (5’-TTTACTAGTACCTGAAAAAGCCCGAAGAGTT-3’) to introduce a Pac1 site upstream of the SA and was inserted into pCR4 (Invitrogen, Karlsruhe, Germany) by TOPO cloning. The SA fragment was recovered by SfiI and SplI digestion and inserted 5’ relative to the adenoviral tripartite leader sequence [37] to yield phM2-2. Subsequently, a fragment encompassing the SV40 splice donor-splice acceptor intron together with the N-terminal part of the humanized GFP (GFP [38]) expression unit [25] was amplified by PCR using primers GFP1F (5’-AAGCGCGCAAGCTTATCGA) and GFP1R (5’-TTTGGCCGAG-3’) using phM2-2 as starting material. The unique AscI and SmaI sites were removed from phM2-2 by filling in with Klenow polymerase followed by ligation. The AscI fragment was used as starting material for gene targeting at the ROSA26 locus we inserted the ade-2b fragment by TOPO cloning. The ade-2b cassette was recovered by BssHII/NotI digestion and ligated into the Pac1 site between the adenoviral tripartite leader SA and reporter gene. The resulting plasmid was then digested using Pac1 and Sma1 and cloned into Pac1 and Sma1 linearized pROSA-PA (kindly provided by S. Srinivas, Department of Physiology, Anatomy and Genetics, Oxford, UK). Plasmid pROSA-PA is a derivative of pROSA26.1 [10] where the unique XbaI restriction site, between exon1 and exon2 is replaced by a Pac1/Sma1/XbaI/Ade2b fragment.
polylinker [8]. The resulting pROSA-hM2 plasmid was linearized with KpnI and subsequently used for electroporation.

**Generation of R26Δ and R26ΔΔ knock-in mice**

The R1 embryonic stem (ES) cell line [39] was electroporated with the linearized pROSA-hM2 targeting vector and G-418-resistant clones were screened for homologous recombination by PCR with the GC-Rich PCR system (Roche, Mannheim, Germany) using primers ROSA-IN (5'-CCTAAAGAAGGGCTGCTTTGG-3') and SA-IN (5'-CATCAAGGAAACCCTGGACTACTG-3'). Furthermore, homologous recombination of ES cells was analyzed by Southern blotting using an 80 nucleotide probe [10] located mainly upstream of the 5' homology region (5' rosa probe) and an 624 nucleotide neomycin probe (neo probe). Correctly recombined ES cell clone 64 was injected into blastocysts to generate chimeric mice. Successful germ-line transmission and correct integration was confirmed by PCR, using primers ROSA-IN and SA-IN. For in vivo excision of the neomycin-resistant cassette, germ-line-transmitting R26Δ knock-in mice were crossed to the deleter-cre strain [11]. Successful excision of the loxP flanked neo/ura cassette was confirmed by PCR. Genotyping was performed using a three primer PCR approach with oligonucleotides ROSA01 (5'-TTCCCTCGTGATCTGCAAACCTCC-3') as forward primer and oligonucleotides ROSA02 (5'-GCTTCAGATGTGCCTTGCTCTC-3') and ROSA07 (5'-CATCAGACTTCTAAGATCAGG-3') as reverse primers under standard PCR conditions, yielding products of 565 bp for the Gt (ROSA)26Sor allele and 916 bp for the R26ΔΔ allele respectively. Gene targeted mice were generated under the licence Az.: 35-9185.81/G-74/07 (Regierungspräsidium Karlsruhe, Germany).

**Animals**

The transgenic mouse lines deleter-Cre, TgPtet-Wnt1-ires-luciferase, TgCamKII-tTA, TgPtetbi-GFP/lacZ and the TgPtetbi-GFP-GluA1/lacZ were administered a solution of doxycycline (DOX, Sigma-Aldrich, Tauferkirchen, Germany) in water containing 1% sucrose. For each experiment the exposure times and DOX concentrations used are specified. DOX treatment was performed under the licence 35-9185.82/A49/06, Regierungspräsidium Karlsruhe. Terminal experiments under the license MPI/T-15/08 and 177-07/981-18 Landesuntersuchungsamt Rheinland-Pfalz.

**RNA isolation and RACE**

Total RNA from mice tissues was homogenized using an Ultra Turrax (IKA, Staufen; Germany) and RNA was isolated with TRI REAGENT® (Molecular Research Center Inc. Cincinnati, OH 45212, USA) followed by phenol/chloroform purification. The 5' RACE was performed using the FirstChoice® RLM-RACE kit (Ambion, Applied Biosystems, Darmstadt Germany) according to the manufacturer instructions. The 5' RLM PCR was performed using in the first reaction the iTA-race out primer (5'-TCTGATGGGCTGTGCACGGTCAAGTG-3') and for the nested PCR the iTA-race in primer (5'-CCCTTCCAGAGGGCCAGAAGTGGTG-3') was used. The PCR products were directly subcloned into the pCR4-toPO vector using TOPO* cloning technology (Invitrogen, Darmstadt, Germany) and subsequently sequenced. Sequences were analyzed using Lasergene (DNAStar.COM; Madison, Wi, USA). Sequences for the RACE iM2 transcripts of the R26ΔΔ allele were annotated to the NCBI database and are available under the accession number HM748862.

**In vivo bioluminescence imaging**

DOX-induced (3 mg DOX/ml for three days) and non-induced compound R26ΔΔ/Ptet-Wnt1-ires-luciferase mice were intraperitoneally injected with luciferin (150 mg/kg body weight, Becton Dickinson, Heidelberg, Germany) and anesthetized by continuous inhalation with isoflurane (Merck, Darmstadt, Germany). After waiting for five minutes to allow distribution of luciferin, the mice were placed in the chamber of an IVIS Lumina optical imaging system (Caliper Life Sciences, Rüsselsheim, Germany) and bioluminescence levels were collected for 15 seconds. The signal intensity was scaled to a pseudocolor image, which was then superimposed on a grayscale photo of the mice using Living Image software v. 3.0 (Caliper Life Sciences, Rüsselsheim, Germany).

**Southern and Western blotting**

Southern blots were performed employing standard procedures using an outside ROSA26 probe as described previously [10] and as an internal neomycin-specific (neo) probe, a 624 bp DNA fragment amplified from the neo gene with the primers rsPneo4 (5'-GGC TATTCGGC TATGAC TGGGC -3') and rsPneo5 (5'-GGGTAGCCCAACGC TATGTC TGG-3').

For Western blot analysis proteins were isolated with an Ultra Turrax in 25 mM HEPES (ph 7,4) containing protease inhibitors (Complete, EDTA-free; Roche, Mannheim, Germany). The homogenates were centrifuged (900 g; 10 min at 4°C) to remove cell debris and 10-25 µg of protein was resolved on a 8-12% SDS-polyacrylamid gel, transferred onto nitrocellulose membranes and probed with anti-GFP (Abcam; ab6556 1:5000, Abcam, Cambridge, UK) and anti-calnexin antibodies (1:3000, Stressgene, Ann Arbor, USA).
Histology and X-Gal staining

For immunohistochemistry the mice were anesthetized with isoflurane (Abbott, Ludwigshafen, Germany) and perfused with PBS/4% paraformaldehyde (PFA, Sigma-Aldrich). The brain was isolated and post fixed in PBS/4% PFA for an additional 3 to 12 hours. For immunohistochemistry, 40- to 70-µm thick vibratom slices were used. Antibody staining on floating vibratom sections was performed as described before [40] using the primary polyclonal anti-GFP primary antibody (Abcam; ab6556 1:5000) together with the anti-rabbit antibody coupled to horseradish peroxidase (Vector Laboratories, 1:600, Vector Laboratories, Eching, Germany) as the secondary antibody. Staining was visualized with 3-3′ dianinobenzidine (DAB, Fluka GmbH, Deisenhofen, Germany), mounted on slides and air-dried. DAB-developed slides were covered with Eukitt mounting medium (Kindler GmbH, Freiburg, Germany). Staining for X-Gal was performed as previously described [41]. All sections were counter-stained with fast red (Sigma-Aldrich, Taukirchen, Germany). Images were captured using a colour view digital camera running on an Olympus BX50 WI microscope. Images were digitalized using the analyzer software package (Soft Image Systems, Münster, Germany) and imported into Photoshop.

Flow cytometry

Cells acquisition was performed on a FACSCalibur or LSRII cytometer (BD) and analyzed using the FlowJo software (Ashland, USA). For lineage determination cells were analyzed as previously described [41]. Before flow cytometry, blood cells from DOX-induced (14 days of 3 mg/ml DOX) or non-induced (normal drinking water) ROSA26-im2 R26ΔCre reporter strains produced by targeted insertion of EYFP into the ROSA26 locus. Cre reporter strains produced by targeted insertion of EYFP into the ROSA26 locus. Mice were generated using the same protocol as described previously [40]. All mice were genotyped before being used for the experiment. The following antibodies were used for staining: c-Kit (2B8), Sca-1 (D7). The CD23 anti-GFP primary antibody (Abcam; ab6556 1:5000) together with the anti-rabbit antibody coupled to horseradish peroxidase (Vector Laboratories, 1:600, Vector Laboratories, Eching, Germany) as the secondary antibody. Staining was visualized with 3-3′ diaminobenzidine (DAB, Fluka GmbH, Deisenhofen, Germany), mounted on slides and air-dried. DAB-developed slides were covered with Eukitt mounting medium (Kindler GmbH, Freiburg, Germany). Staining for X-Gal was performed as previously described [41]. All sections were counter-stained with fast red (Sigma-Aldrich, Taufkirchen, Germany). Images were captured using a colour view digital camera running on an Olympus BX50 WI microscope. Images were digitalized using the analyzer software package (Soft Image Systems, Münster, Germany) and imported into Photoshop.

Statistical analysis

For statistical data analysis the two-tailed t test was applied using the Prism4 package for Windows (GraphPad software, La Jolla, USA).

Acknowledgements

We thank L.A. Chodosh for providing the Ptet-Wnt1-ires-luciferase responder mouse strain. We also thank the animal technicians of the Mainz mouse facility for excellent assistance and mouse care. Finally, we are grateful to Peter H. Seeburg for his constant support and to An Waisman enabling us to finish the work in his lab. This study was supported by the Deutsche Forschungsgemeinschaft (Forschergruppe 527; „Suszeptibilitätsfaktoren der Tumorgene“, SFB636/4A), the VW foundation, the Dr. Mildred Scheel Stiftung für Krebsforschung (10-1982-80) and the Nationlen Genomforschungsinstitut (NGFN).
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