

TECHNOLOGY REPORT

A Mouse Tool for Conditional Mutagenesis in Ovarian Granulosa Cells

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Summary: Here we describe the generation of an inducible Cre transgenic line allowing conditional mutagenesis in ovarian granulosa cells. We have expressed the tamoxifen inducible CreER^{T2} fusion protein from a Bacterial Artificial Chromosome (BAC) containing the regulatory elements of the hydroxysteroid (17- β) dehydrogenase 1 (*Hsd17b1*) gene. *Hsd17b1-iCreER^{T2}* transgenic mice express the iCreER^{T2} fusion protein exclusively in ovarian granulosa cells. Recombination analysis at the genomic DNA level using mice with “floxed” *Stat3* alleles showed no Cre activity in absence of tamoxifen whereas tamoxifen treatment induced Cre activity solely in the ovaries. Further characterization of *Hsd17b1-iCreER^{T2}* mice using a Cre reporter line demonstrated that Cre-mediated recombination was restricted to ovarian granulosa cells. Therefore, *Hsd17b1-iCreER^{T2}* mice should be a useful tool to analyze the gene functions in ovarian granulosa cells. *genesis* 48:612–617, 2010. © 2010 Wiley-Liss, Inc.

Key words: iCreER^{T2}; loxP; tamoxifen; *Hsd17b1*; eGFP; *Stat3*

Conditional mutagenesis has become a versatile tool to study the gene functions in vivo. The use of site-specific recombinases allows precise genome engineering such as inactivation or activation of genes, large chromosomal deletions, inversions, and translocations (Branda and Dymecki, 2004; Kwan, 2002; Tronche *et al.*, 2002). In particular, the Cre/loxP system is widely used in mouse genetics. The Cre recombinase is an enzyme derived from the bacteriophage P1 that catalyzes the recombination between two 34 bp sequences named loxP (Abremski *et al.*, 1983). The Cre/loxP system can be used to inactivate or activate a gene in a cell type in a temporally-controlled manner (Branda and Dymecki, 2004; Kwan, 2002; Tronche *et al.*, 2002). The Cre/loxP system is most commonly applied by combination of two elements: a transgenic line expressing the Cre recombinase under a tissue specific promoter and a second transgenic line harboring a gene of interest which is flanked by two loxP sites. By crossing both lines, inacti-

vation of the desired gene will occur only in cells expressing the Cre recombinase. Alternatively, the second transgenic line may harbor a gene that is not expressed because of a transcriptional stop cassette flanked by two loxP sites. Crossing these mice with a Cre-expressing line allows the activation of the gene of interest in a tissue specific manner. Ideally, the Cre recombinase activity should be controllable in a time-dependent manner, thus allowing the inactivation or activation of a gene at a desirable time point. The Cre recombinase can be controlled at the transcriptional level using the Tetracycline system (Schonig *et al.*, 2002). Another common method is to fuse the Cre recombinase to the ligand binding domains (LBD) of steroid hormone nuclear receptors, such as estradiol, progesterone, androgen, or glucocorticoid receptors (Brocard *et al.*, 1998; Kellendonk *et al.*, 1996; Metzger *et al.*, 1995; Shimshek *et al.*, 2002). Such fusion proteins are bound to heat shock proteins, which maintain the Cre fusion protein in an inactive conformation in the cytoplasm. Upon binding of the ligand to their respective LBD, the heat shock protein complex is released and the Cre fusion protein can translocate into the nucleus, where it can catalyze DNA recombination.

Two lines expressing the Cre recombinase in ovarian granulosa cells have been described: the anti-Müllerian hormone Cre (AMH-Cre) expressing the cre recombinase in granulosa and sertoli cells (Lecureuil *et al.*, 2002) and the *Cyp19-Cre* (Fan *et al.*, 2008). However, none of these lines allows the temporal control of the Cre activity. We have generated a transgenic line expressing the

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iCreER^{T2} fusion protein in ovarian granulosa cells that allows tamoxifen induction of Cre recombinase.

A screen of a gene expression database (<http://biogps.gnf.org/>, (Su *et al.*, 2004)) for genes whose expression was enriched in ovarian tissue revealed the hydroxysteroid (17-beta) dehydrogenase 1 (*Hsd17b1*) gene as a good candidate. *Hsd17b1*, an enzyme implicated in ovarian estradiol biosynthesis (Nokelainen *et al.*, 1996), has been previously shown to be expressed solely in ovarian granulosa cells (Nokelainen *et al.*, 1996; Pelletier *et al.*, 2004), thus making the regulatory elements of this gene suitable to direct gene expression to ovarian granulosa cells.

To generate a vector to express the iCreER^{T2} fusion protein in ovarian granulosa cells a Bacterial Artificial Chromosome (BAC) containing the *Hsd17b1* locus was modified through homologous recombination in *E. coli* (Zhang *et al.*, 1998) by inserting a cassette encoding the iCreER^{T2} into the first exon of the *Hsd17b1* gene (Fig. 1a). Three transgenic lines were obtained by pronuclear oocyte injection of the *Hsd17b1-iCreER^{T2}* BAC. Southern blot analysis of the transgenic lines showed that lines 1 and 2 harbor 2 copies, while line 3 contains approximately eight copies of the *Hsd17b1-iCreER^{T2}* BAC (Fig. 1b). mRNA expression analysis revealed that line 3 had the highest iCreER^{T2} expression levels in ovaries (Fig. 1c), therefore, this line was used for further analysis. To determine the ovarian cell type which expresses the Cre recombinase, we performed in situ hybridization analysis in ovaries from wild-type and *Hsd17b1-iCreER^{T2}* mice using a probe against the iCre mRNA. As expected, we did not detect any Cre expression in wild-type mice while in *Hsd17b1-iCreER^{T2}* mice, the expression was restricted to the ovarian granulosa cells (Fig. 1d).

To test the recombination pattern, we crossed of the *Hsd17b1-iCreER^{T2}* mice with mice harboring a "floxed" allele for the *Stat3* gene (Alonzi *et al.*, 2001). *Stat3* flox mice harbor exons 12, 13, and 14 (a 2kb region containing the putative DNA binding domain) flanked by two loxP sites. PCR analysis of genomic DNA from different organs of double transgenic mice treated with tamoxifen showed a PCR product for the recombined *Stat3* allele only in the ovaries (Fig. 2a). Importantly, no recombination was observed in untreated double transgenic mice. To gain insight into the spatial pattern of recombination, we crossed the *Hsd17b1-iCreER^{T2}* mice with a eGFP Cre reporter line [*CAG-CAT-EGFP* (Kawamoto *et al.*, 2000)]. Immunohistochemistry against eGFP in tamoxifen-treated double transgenic mice indicated that the *Hsd17b1-iCreER^{T2}* mice recombine only in ovarian granulosa cells. No eGFP staining was observed in double transgenic mice in absence of tamoxifen (Fig. 2b).

Despite the broad expression of the iCreER^{T2} in granulosa cells tamoxifen-induced recombination was not observed in all the cells using the Cre reporter strain. This could be due to mosaic expression of the reporter gene or due to the reduced activity of the iCreER^{T2} fusion protein (Casanova *et al.*, 2002).

One possible application of the *Hsd17b-iCreER^{T2}* mice is the analysis of a given gene in fertility studies. It has been reported that Cre activity might be toxic in some contexts (Silver and Livingston, 2001). To rule out the possibility that Cre activity may affect fertility, we treated four wild-type and 4 *Hsd17b1-iCreER^{T2}* females with tamoxifen (five consecutive days, 1 mg injected intraperitoneally) and mated them with wild-type males after seven days. The average time for the first litter was of 47.5 ± 8.6 days in the case of wild-type females and 52.5 ± 9.3 days for the *Hsd17b1-iCreER^{T2}* females. The average size of the litter was 4.2 ± 2.2 and 3.3 ± 0.9 for wild-type and mutant females, respectively. These results suggest that *Hsd17b1-iCreER^{T2}* females do not show reduced fertility upon tamoxifen treatments compared to wild-type females and they are suitable for fertility analysis.

In summary, the *Hsd17b-iCreER^{T2}* mice might represent a valuable tool to study gene functions in granulosa cells, fertility studies, or analysis of the role of oncogenes and tumor suppressor genes to the development of granulosa cell tumors. Furthermore, the *Hsd17b1* BAC represents a useful tool to express genes of interest in ovarian granulosa cells in vivo.

MATERIALS AND METHODS

Generation of *Hsd17b1-iCreER^{T2}* Mice

A 200 kb Bacterial Artificial Chromosome (RP24-178B1) harboring the *Hsd17b1* gene was purchased from Children's Hospital Oakland Research Institute. To generate the *Hsd17b1-iCreER^{T2}* BAC the open reading frame of the iCreER^{T2} (Casanova *et al.*, 2002), a fusion protein between an improved Cre recombinase version (Shimshak *et al.*, 2002) and a mutated form of the human ligand binding domain of the estrogen receptor (Feil *et al.*, 1997), was inserted into the exon containing the transcription initiation codon of the *Hsd17b1* gene via homologous recombination in *E. coli* as previously described (Casanova *et al.*, 2001). Briefly, a targeting construct containing a 200 bp 5'homology region to the upstream part of the exon harboring the initiation codon of the *Hsd17b1* gene (PCR amplified with the oligos P₁ 5'ACAAACAGCACCCTTTGGTGCTTTG and P₂ 5'AGTCAGCAGGTTGGACATGTGGAATAGTACTAAAGTTCAGAGC), the iCreER^{T2} open reading frame, a bovine growth hormone polyadenylation signal, an ampicillin marker flanked by two FRT sites and a 200 bp 3'homology region to the downstream area of the targeted exon (PCR amplified with the oligos P₃ 5'GTGCTCATACCGGTTGCTCCTCTG and P₄ 5'TGTGCCTTCAGGTCCCAGCAATG), was assembled using conventional cloning techniques. A 50-ml culture of *E. coli* harboring the *Hsd17b1* BAC and the pSC101-BAD-gba-tet plasmid (Zhang *et al.*, 2003) was grown at 30°C in presence of 12.5 µg/ml chloramphenicol and 3 µg/ml tetracycline until the optical density (OD) reached 0.2. At this point, L-arabinose was added to the culture (0.2%, final concen-

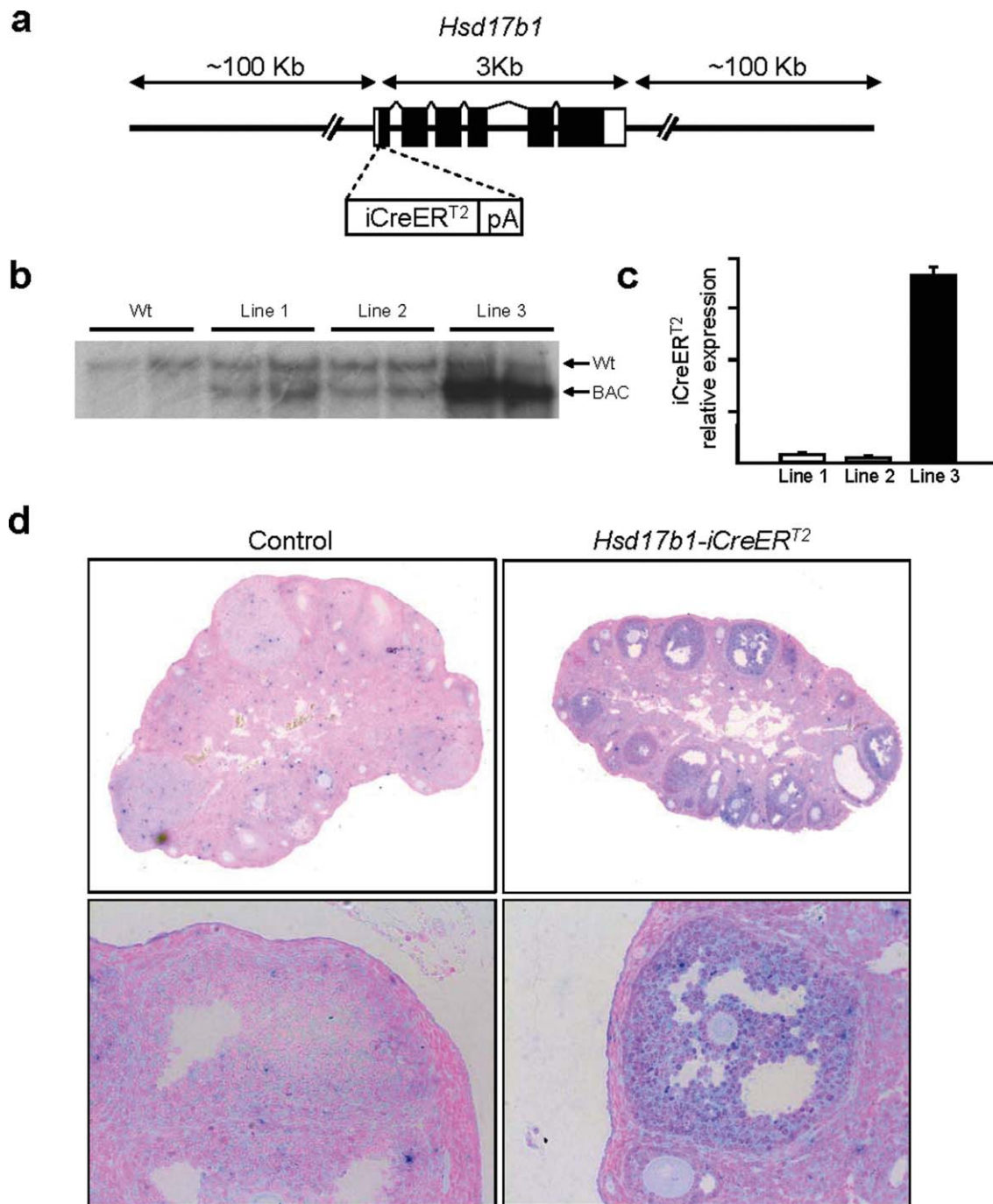


FIG. 1. Generation of the *Hsd17b1-iCreER^{T2}* transgenic mice. **(a)** Schematic representation of the *Hsd17b1-iCreER^{T2}* construct; a 200 kb BAC containing the *Hsd17b1* gene was modified by homologous recombination in *E. coli*. A construct encoding the open reading frame of the iCreER^{T2} and a bovine growth hormone polyadenylation signal (pA) was inserted into the exon harboring the initiation codon of the *Hsd17b1* gene. White rectangles represent the 5' and 3' untranslated regions; black rectangles represent the coding exons of the *Hsd17b1* gene. **(b)** Copy number analysis of the *Hsd17b1-iCreER^{T2}* transgenic lines; Genomic DNA from three independent lines generated with the *Hsd17b1-iCreER^{T2}* BAC was digested with *SacI*, blotted and hybridized with an external probe that binds the endogenous *Hsd17b1* locus (Wt, 13.2 Kb) and the *Hsd17b1-iCreER^{T2}* BAC (BAC, 9 Kb). Lines 1 and 2 harbor 1 or 2 copies, and line 3 contains approximately 8 copies of the *Hsd17b1-iCreER^{T2}* BAC. **(c)** Quantification of the iCreER^{T2} expression on mRNA level; Total RNA from ovaries of the transgenic lines 1, 2, and 3 was isolated and the cDNA retro-transcribed. Relative expression levels were analyzed using quantitative real time PCR with specific oligos amplifying iCreER^{T2} cDNA and *Gapdh* as internal control. **(d)** *Hsd17b1-iCreER^{T2}* transgenic mice express the iCreER^{T2} in granulosa cells of the ovarian follicles. We performed in situ hybridization with an antisense probe against the iCre recombinase in ovaries of control animals (wild-type) and *Hsd17b1-iCreER^{T2}* transgenic mice. We could detect a specific signal in ovarian granulosa cells of the *Hsd17b1-iCreER^{T2}* transgenic mice but not in wild-type animals. An iCre recombinase sense probe did not detect any signal in both genotypes (data not shown). 25 \times (upper panel) and 200 \times lower panel magnification.

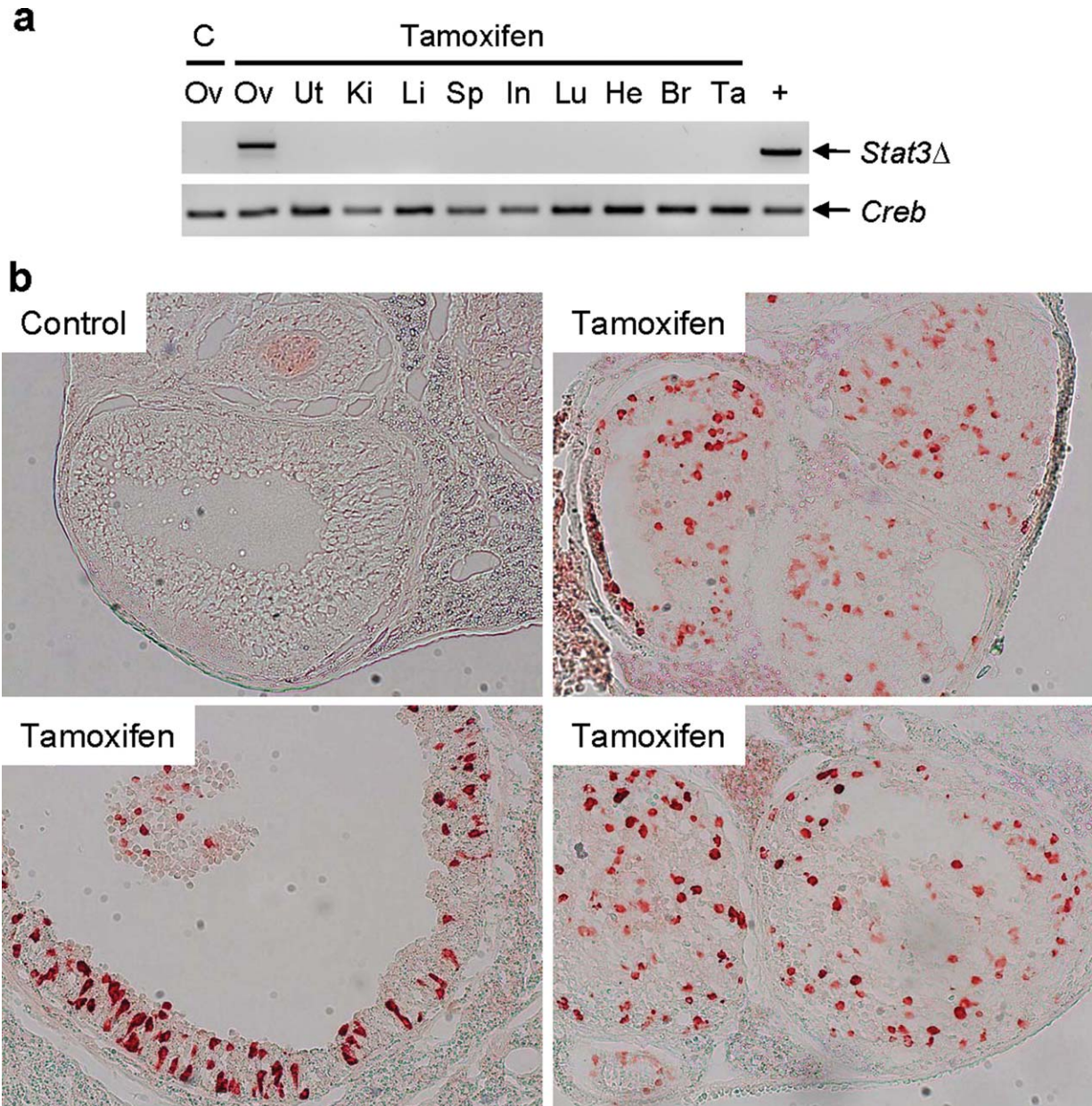


FIG. 2. Recombination analysis of the *Hsd17b1-iCreER^{T2}* transgenic mice. **(a)** *Hsd17b1-iCreER^{T2}* transgenic line recombines only in ovaries. Eight-week-old female, double transgenic mice harboring the *Hsd17b1-iCreER^{T2}* and the *Stat3^{loxP/loxP}* alleles were intraperitoneally injected five times with 1 mg of Tamoxifen (or sun flower oil in the negative controls) and analyzed one week after the last injection. Genomic DNA from different organs was isolated and the recombined *Stat3* allele (*Stat3^Δ*) was PCR amplified. As loading control we PCR amplified a genomic region of the *Creb* gene. C, Negative control, injected with sunflower oil; Ov, Ovary; Ut, Uterus; Ki, Kidney; Li, Liver; Sp, Spleen; In, Small intestine; Lu, Lung; He, Heart; Br, Brain; Ta, Tail; +, positive control, tail genomic DNA from a heterozygous *Stat3^{Δ/+}* mouse. **(b)** Recombination in the *Hsd17b1-iCreER^{T2}* strain is restricted to granulosa cells of the ovarian follicles. 8-week-old female, double transgenic mice (*Hsd17b1-iCreER^{T2}*; *CAG-CAT-EGFP*) were intraperitoneally injected five times with 1 mg of Tamoxifen (or sun flower oil in the negative controls) and analyzed one week after the last injection. Recombination was visualized by immunohistochemistry using an antibody against eGFP. $\times 200$ magnification.

tration) and the culture was shifted to 37°C until the OD reached 0.5 and electrocompetent cells were prepared. Cells were electroporated with 1 μ g of the linearized targeting construct mentioned above and seeded overnight at 37°C in the presence of 50 μ g/ml ampicillin and 12.5 μ g/ml chloramphenicol. Clones harboring correctly recombined BACs were identified by Southern blot and

direct sequencing. The ampicillin cassette present in the BAC, flanked by FRT sites, was removed upon electroporation of a Fip expressing plasmid [706-Fip, (Buchholz *et al.*, 1996)]. Totally, 50 μ g of the modified *Hsd17b1-iCreER^{T2}* BAC digested with NotI was applied to a self-made column filled with Sepharose CL-4b (Pharmacia) which had been pre-equilibrated with injection buffer

(10 mM Tris/HCl pH7.4, 0.1 mM EDTA, 100 mM NaCl) (Yang *et al.*, 1997). Fractions of 0.5 ml were collected and the BAC integrity was analyzed using pulsed field gel electrophoresis. BAC DNA was pronuclear injected at 1 ng/ μ l in B6CBAF1-derived oocytes. *Hsd17b1-iCreER^{T2}* mice were genotyped using the oligos: P₅ 5'AGGA TTGGGAAGACAATAGCAGGC and P₆ 5'GATTTGGAGT CTCTGACATCCAATTCC.

Copy Number and mRNA Expression Analysis

Tail genomic DNA (10 μ g) of transgenic and wild type mice (F1) was digested with SacI, fractionated in a 1% garose gel, transferred to a Nylon membrane (GeneXpress), and hybridized with an external probe (PCR amplified using oligos P₇ 5'AGGACCGCCTACCTGAAGCAAGAG and P₈ 5'TCAGGGACAATTATAACTACCAG) located upstream of the insertion site of the *iCreER^{T2}* construct.

Total RNA from ovaries of transgenic mice was isolated with the RNeasy Mini Kit (Qiagen). cDNA was prepared using the oligo-(dT) RevertAid H Minus First Strand cDNA synthesis kit (Fermentas). For quantification of mRNA expression, we performed quantitative real time PCR using 1 μ g of cDNA with the oligos P₉ 5'CTGCCAGAGTGGGTGCTGCCAGGG and P₁₀ 5'CAT CTGGTCGGCCGTCAGGGACAAGGCC (detecting the *iCreER^{T2}*) and normalized mRNA levels to the *Gapdh* gene (oligos P₁₁ 5'AGAAGGTGGTGAAGCAGGCATC and P₁₂ 5'CGGCATCGAAGGTGGAAGAGTG).

Tamoxifen Administration

Tamoxifen free base (Sigma T5648) was dissolved in sun flower oil (Fluka) at 10 mg/ml by continuous rotation at room temperature. Mice were injected intraperitoneally for five consecutive days with 1 mg of Tamoxifen.

Recombination Analysis

Genomic DNA isolated from different organs was analyzed for deletion of the *Stat3* "floxed" gene using a PCR strategy (P₁₃ 5'GCAGCAGAATACTCTACAGCTC and P₁₄ 5'CACCAACACATGCTATTTGTAGG) that detects the recombined *Stat3* allele (Alonzi *et al.*, 2001). As loading control we used a genomic region of the *Creb* gene (P₁₅ 5'CATCTCCTACAGACTAACATGATGCC and P₁₆ 5'TAT AGACTCATAACTCATGTCGGAATCC).

Immunohistochemistry

Ovaries were fixed in 4% PFA overnight at 4°C. For eGFP immunohistochemistry, fixed ovaries were transferred to sucrose (20% in PBS), stored overnight at 4°C, and snap-frozen in isopentane/liquid nitrogen. Samples were embedded in Tissue-Tek (Sakura Finetek). Totally, 8- μ m sections were cut using a Microm HM500 OM Cryostat and mounted onto SuperFrost slides (Thermo Scientific). Immunohistochemistry was performed with a rabbit polyclonal anti-GFP antibody (A11122, Invitrogen, 1:1,000) and the immunoreactivity was detected using the avidin-biotin system (Vectastain, Vector Labs).

In Situ Hybridization

Paraffin sections (2 μ m) were hybridized with in vitro transcribed sense and antisense digoxigenin (DIG -11-UTP) (11209256910, Roche) labeled RNA probes against the *iCre* recombinase in hybridization solution (10 mM Tris, pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.25% SDS, 10% dextran sulfate, 1x Denhardt's buffer, 200 μ g/ μ l yeast tRNA, 50% formamide) at 65°C. Anti-digoxigenin-AP, Fab fragments (11093274910, Roche) were used to detect the digoxigenin labeled RNA probes. Color development was carried out using BM purple substrate (11442074001, Roche), followed by counter-staining with 0,05% nuclear fast red (N069.1, Carl Roth GmbH).

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