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CUSTOMIZED TRANSGENESIS VIA MODIFICATION OF SPERMATOGONIAL STEM CELLS

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ABSTRACT

Spermatogonia mediated transgenesis is becoming an increasingly popular method of genetic modification in animals. It guarantees direct germline transmission because spermatogonial stem cells are the unipotent type of stem cell and can differentiate only into the mature spermatozoa. Additionally, modified spermatogonial stem cells can be clonally expanded. Clone or several clones can be specifically identified for the presence of the desired alteration, expanded *in vitro* and transplanted *in vivo*.
Since expression of DAZL gene as well as DAZL promotor driven transgene is restricted to germ cell line, we expected that DAZL promoter-driven transgenic tamoxifen inducible ERT2CreERT2 can be utilized for the germline-specific controllable Cre mediated recombination of targeted DNA flanked by loxP sites.
Here we describe a transposon-mediated transformation of spermatogonial stem cells and generation of transgenic rat line, DAZL-ERT2CreERT2 facilitated by transplantation of modified cells into testis of DAZL deficient recipients-producers. In obtained animal line tamoxifen inducible engineered ERT2CreERT2 recombinase is specifically expressed in germ cells at a level sufficient for ligand-depended efficient recombination. Using male germ cells from a generated animal and its cross to GCS-EGFP transgenic rat, several reporter spermatogonial cell lines were prepared to test the performance of the transgene *in vitro* and *in vivo*.
This transgenic rat line can be utilized not only for analysis of the genetic makeup of spermatogenesis but potentially for germline activation and transmission of desirable ERT2CreERT2 mediated rearrangements of tissue-specific genetic elements flanked by loxP sites located in the part of the spermatogonial genome, accessible for the recombinase.

Keywords: gene, promotor, transgenesis, spermatogonial stem cells, transgenic rat

INTRODUCTION

Traditionally an investigation of gene function in human relies on studying of naturally occurring mutant genotypes manifested in phenotypes. Due to obvious ethical reasons, such studies are limited and most of the related research is done in rodent models. Artificial generation of a large number of random mutations using chemical or radioactive agents resulted in big collections of phenotypes and greatly expanded researcher's abilities to study a genetic background of the related condition. However, not all random mutations can result in an observable phenotype and therefore could be overlooked. Retroviral and transposon-based insertional mutagenesis provided the ability to tag and define the position of the foreign DNA integration enabling an identification of a mutant though still randomly generated **(Howard et al., 2010; Ivics et al, 2011; Furushima et al 2012).**
Application of homologous recombination (HR) in rat embryonic stem cells was a significant step forward in gaining control over the genome modification process as it allows creating predetermined stable alterations in a specifically targeted DNA locus **(Kawamata et al., 2010; Tong et al., 2011)**. Combination of the insertional transgenesis or endogenously facilitated homologous recombination with exogenous enzyme-mediated site-specific recombination systems such as Cre/LoxP, FLP/FRT, φC31/att provides an additional control over the modified fragment of DNA by triggering its activity **(Birling et al., 2009).** At present Cre/LoxP system is most widely utilized one among currently available recombination systems. Choice of tissue or cell-type specific promoters to drive an expression of Cre recombinase provides spatial control over its expression. Further improvement of the system was the achievement of timely regulation the recombinase activity. By fusing N and C terminus of Cre protein to hormone binding domains of estrogen receptor an enzyme was engineered in such a way that now it can be activated by estrogen antagonist 4-hydroxytamoxifen in a ligand-dependent manner while remaining unresponsive to the endogenous level of estrogen **(Feil et al., 1997).**
Because of amenability of pronuclear injection, a mouse, among vertebras, was a subject of choice for transgenesis in general and for the creation of Cre driver lines in particular. The CREATE database (http://creline.org/) lists over two thousand driver lines for different Cre variants in a variety of tissues. Due to the larger size and physiological closeness to humans, rats are more favorable and preferable for many studies and experiments. However, because of difficulties with rat transgenesis, less than a dozen Cre models are established for this specie. Spermatogonia mediated genetic modification offers attractive and efficient alternative to embryo-based transgenesis **(Brinster, 1994; Brinster, 2002**). Our laboratory was an established expert in spermatogonial stem cell-mediated rat transgenesis **(Chapman et al., 2011)**. Usage of proven technology allowed us to bypass complication of microinjection, embryo manipulation and breeding chimeras **(Kanatsu-Shinohara et al, 2008; Takehashi et al.; 2010; Chapman et al., 2011).**
Majority of the Cre lines were generated with the goal to spatially control recombinase activity in tissues and cell types which are of the prime interest to investigators. Since scientific interest and research efforts of our team was focused on rat spermatogenesis we set up to generate germ-cell-specific Cre line using currently the most advanced form of engineered enzyme - ERT2CreERT2 **(Matsuda and Cepko, 2007)**. DAZ (Deleted in AZoospermia) end related proteins (DAZL, BOULE) expression is restricted to rat germ cell lines of both sexes in a variety of species. It was demonstrated previously that promoter sequence of this RNA binding protein can effectively and specifically drive expression of GFP in germ cells in mice and rats (Reijo et al, 2000; Nicholas et al., 2004;). We rationalized that analogous rat promoter can be used to drive expression of ERT2CreERT2 in rat germ cells. The objective of our study was to demonstrate a feasibility to utilize spermatogonial stem cell for generation of customized rat transgenic model with desirable traits.

MATERIAL AND METHODS

Constructs preparation

A backbone of Sleeping Beauty transposable element was used to clone-in 2138bp of DAZL promoter sequence (amplified by PCR from germ cell cDNA) to construct parental vector SB-DAZL-MCS**.** Using Gateway® Vector Conversion System a Gateway® cassette containing attR recombination sites

flanking a ccdB gene and chloramphenicol resistant gene we converted the parental vector into a destination vector SB-DAZL-GW by blunt-end ligation of the recombination cassette into multiple cloning site of the parental vector. pCAG-ERT2CreERT2 was a gift from Connie Cepko (Addgene plasmid # 14797). The sequence coding for ERT2CreERT2 fusion protein was PCR amplified from this vector (using following primers: Forward primer – CACCGGTCGCCACCATGCCAATTTACTG; Reverse primer – AGCTGTGGCAGGGAAACCCTCTG. Purified PCR product was cloned by the directional TOPO® cloning kit (Invitrogen) into pENTR/D-Topo resulting in pENTR/D-Topo-ERT2CreERT2 entry vector. From the entry vector, the ERT2CreERT2 fragment was transferred to destination vector SB-DAZL-GW by the Gateway® LR reaction thus generating expression plasmid SB-DAZL-ERT2CreERT2. Additionally, we used pM3A vector expressing transposase a generous gift from Dr. Izsvak laboratory. For positive selection of transformed cells, the pNeoΔTK vector was prepared from pNeoTK (Invitrogen) by deletion of TK cassette.

The reporter plasmid pCALNL-GFP was a gift from Connie Cepko (Addgene plasmid # 13770) and in described instances was utilized directly, without modifications. Plasmid Pgk-Cre was a generous gift from Erik Olson laboratory and was used as positive control in test transfections and during functional analysis. SB-DAZLenCAG-LoxNeoLox-GFP was constructed using backbone of parental vector SB-DAZL-MCS and CAG-LoxNeoLox-GFP cassette excised from the pCALNL-GFP vector with SalI and NotI restriction enzymes. SB-DAZLenCAG-LoxNeoLox-Tomato and SB-DAZLenCAG-LoxNeoLox-DNERBB2 were constructed from SB-DAZLenCAG-LoxNeoLox-GFP by removing GFP sequence with SwaI and NotI restriction enzymes followed by blunting and dephosphorylating the vector. Corresponding PCR amplified DNA sequences were cloned by blunt-end cloning with following verification of fragment orientation by the enzymatic restriction analysis and sequence integrity by Sanger DNA sequencing.

Testing the SB-DAZL-ERT2CreERT2 transgene *in vitro*

To estimate the functionality of SB-DAZL-ERT2CreERT2 construct *in vitro* 1:3 molar ratio of SB-DAZL-ERT2CreERT2 plasmid and pCALNL-GFP reporter vector were transiently lipofected into COS cell in two separate wells. Plasmid PGK-Cre with the active form of Cre protein was used as a positive control at the same molar ratio, lipofection with pCALN-GFP only served as a negative control. After 24 hours, one well with SB-DAZL-ERT2CreERT2 transfected cell were supplemented with medium containing 1μM of 4-hydroxytamoxifen. The efficiency of the inducible Cre mediated recombination was assessed by the observation of expression of GFP protein under the Olympus inverted microscope using UV light.

Cell culture

Freshly isolated lamining binding population of germ cells from analyzed pups was prepared by previously established method **(Chapman et al., 2011).** Proliferating spermatogonial cell lines were maintained in SG medium published earlier **(Wu et al., 2009).** DR4 MEF cells from Biopioneer were used as feeder layer cells for the cultured spermatogonia.

Transformation of T138 rat spermatogonial line with SB-DAZL-ERT2CreERT2, selection, and expansion of transformants

To generate a sufficient amount of transformed cells two electroporation with 1.8 E6 of spermatogonial cells each were conducted using Nucleofector® device (Lonza) and Cell Line Nucleofector Kit L (Lonza). 7.5μg of SB-DAZL-ERT2CreERT2 plasmid, 5μg of pNeoΔTK plasmid and 2.5μg of M3A plasmid were resuspended in the solution L in a microcentrifuge tube to a total volume of 60μl and incubated for 5 min at room temperature. A pellet containing 3.6 E6 cells of freshly harvested T138 proliferating rat spermatogonial line was split into two parts. Each part was resuspended in 75μl of the solution L and transferred to a separate centrifuge tube. 30μl of plasmid cocktail was added to each cell suspension and incubated for 5 min before transfer to the Amaxa electroporation cuvettes. After executing the A-020 program by the Nucleofector® device, the cuvettes were filled with pre-warmed gem cell medium. Transformed cells were collected with a pipet provided with the kit and plated on freshly prepared MEFs with fresh SG media without the supplement of the antibiotic-antimycotic mixture. SG medium with antibiotic-antimycotic was applied next day and was changed every two days. Selection of transformed cells with SG medium containing 60mg/ml of G418 (Invitrogen, Inc.) started on day 3 post-transfection and continued for 4 more feedings. To expand the number of modified and selected spermatogonia the cells were propagated for three more passages. On the day of transplantation 4.3 mln cells were harvested from 3/10 cm culture dishes and plated on the gelatin-coated plate for 40 min to reduce the number of nonspermatogenic cells and contaminating MEFs. Total 2.9 mln cells were collected after gelatin selection and resuspended in 360 ml of cold SG media ready for transplantation.

Generation of chimeric Recipient-Producers by transplantation of genetically modified spermatogonia

Two home-bred male-sterile DAZL-deficient pups **(Dann et al., 2006)** at 12 days of age were treated with a single dose of busulfan (12 mg/kg) in order to deplete endogenous population of spermatogonia and enhance engraftment of T138 spermatogonia line transformed with SB-DAZL-ERT2CreERT2 . At 24 days of age, animals were transplanted with 400K of modified cells per testis according to the established protocol. Upon reaching reproductive maturity animals with transplanted spermatogonia became recipient-producers capable of generating genetically modified spermatozoa and were mated to WT Sprague Dawley female rats.

Initial screen of line founders by functional analysis

A single (left) testicle was removed from each F1 male pup at age of 22-24 days during the survival surgery. Primary cell cultures were prepared from each testicle to correspond a respective animal. Typically 150K-300K of Laminin binding cells per testicle was obtained. Immediately after isolation cells were counted and split into two equal parts; one part maintained as a control for the second part being electroporated with pCALNL-GFP using Neon® Transfection System. Usually, 1μg of plasmid DNA was mixed with 100K of laminin binding spermatogonia in 20μl of buffer R. Cells were electroporated twice using 10μl Neon® pipet tip with two pulses per electroporation at 1120 V for 20 msec. Each fraction of electroporated cells was plated on freshly prepared MEFs and supplemented with SG medium without an antibiotic-antymicotic mix. On the following day, cells were fed with complete SG medium. SG medium with 4-hydroxytamoxifen was added to the test well.

Generation of double and triple transgenic reporter cell lines.

The Neon® Transfection System (Life Technologies) was used to transform spermatogonia for the generation of double and triple transgenic reporter cell lines. Typically 3 E6 cells of freshly harvested proliferating rat spermatogonia from DAZL-ERT2CreERT2 line #1 or cross between DAZL-ERT2CreERT2 line #1 and GCS-EGFP (Germ Cell-Specific EGFP) transgenic rat **(Cronkhite et al., 2005)** were resuspended in 300μl of the buffer R containing 25μg of the Sleeping Beauty based reporter vector (mentioned above) and 5μg of the vector carrying Sleeping Beauty transposase. Using a single 100μl Neon® pipette tip cells were electroporated with two pulses at 1100V / 20ms. Transformed cells were resuspended in warm antibiotic free SG medium and plated on freshly prepared MEFs with fresh SG media without the supplement of the antibiotic-antimycotic mixture on 6 well plate. SG medium with antibiotic-antimycotic was applied next day and was changed every two days. Selection of transformed cells with SG medium containing 60mg/ml of G418 (Invitrogen, Inc.) started on day 3 post-transfection and continued for 4 more feedings. After the initial selection and expansion part of the transfected cells was plated on 3 well of the 6-well plate at the density of 75K /plate for the colony pick-up. From 100 to 300 colonies per transformation were manually picked up under the Leica MZ9.5 high-performance stereomicroscope.

4-hydroxytamoxifen doses and treatment

During vectors analysis in Cos cell 1mM of 4-hydroxytamoxifen (Sigma T176) was used. After flow sort analysis of the time-dose response of DAZL-ERT2CreERT2 line #1 germ cells (modified with SB-DAZLenCAG-LoxNeoLox-EGFP) to 4-hydroxytamoxifen, the dose of 100nM was estimated as optimal and used thereafter. For the *in vivo* induction of recombination, we used two i.p. injections at 60mg/kg twice with three days interval. For this purpose, the 4-hydroxytamoxifen was resuspended in refine sunflower oil of Ukrainian origin obtained from Wallmart superstore.

RESULTS AND DISCUSSION

The transgenic construct was generated using GW cloning system (Life Science). The PCR product of ERT2CreERT2 fusion protein was amplified from Addgene plasmid #13777 and cloned into a donor vector. The DNA fragment of DAZL promoter in front of GateWay recombination cassette was assembled in a backbone containing Inverted Repeats of Sleeping Beauty transposable element and was termed SB-DAZL-GW (destination vector). Upon the LR recombination reaction between donor and destination vectors sequence coding for ERT2CreERT2 was placed in front of DAZL promoter thus yielding expression vector.

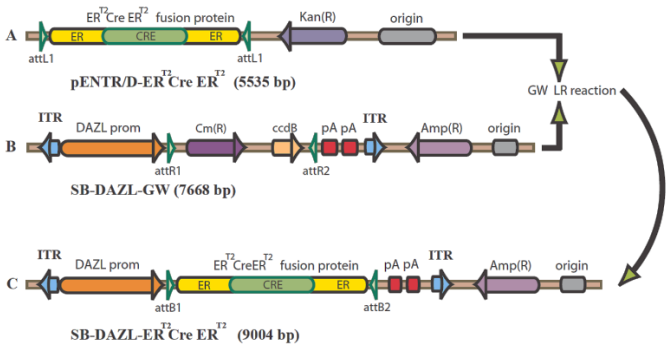

Figure 1 Construction of the SB-DAZL - ERT2CreERT2 transposable DNA construct. A) pENTR/D-Topo donor vector with cloned sequence for ERT2CreERT2 fusion protein; B) SB-DAZL-GW destination vector with GateWay recombination cassette; C) SB-DAZ L- ERT2CreERT2 expression vector as a result of LR recombination reaction between the donor and the destination vectors.

It worth mentioning that ERT2CreERT2 sequence in the final construct is flanked by short attB sites providing a potential for further manipulations. Several clones of the generated construct were preliminary tested in the COS cell line for the performance and response to tamoxifen treatment using the reporter plasmid pCALN-GFP as a target for the recombination. Even though DAZL promoter is not optimal for protein expression in COS cells functionality of the construct was confirmed by observation of the GFP expression as a result of the recombination event. The highly purified and concentrated SB-DAZL-ERT2CreERT2 plasmid along with the pM3A plasmid coding for Sleeping Beauty transposase and the plasmid pKONΔTK containing Neomycin resistant gene was introduced by electroporation into spermatogonial line T-138 at passage 12 (Fig 2, A). Due to its nature Sleeping Beauty mediated transgenesis has mutagenic potential. To reduce the possibility of random mutagenesis plasmids ratio of Sleeping Beauty transposition complex was formulated with an intention to achieve possible low to single transgene integration per genome **(Ivics et al., 2011; Furushima et al., 2012)**. The pKOΔTK plasmid provided a transient Neomicyne resistance of modified cells necessary for the selection of transformants. Electroporated cells underwent Neomycin selection during one passage and were expanded for three more passages. Transformed spermatogonia were harvested and transplanted into testes of two busulfan treated DAZL deficient male rat recipients **(Hirayanagi et al., 2015).** After the transplantation, each of the transformed cells has a potential to expand clonally and generate viable sperm cells. Upon reaching reproductive maturity, the recipients with modified germ cells became chimeric producers of modified spermatozoa and were mated with WT Sprague Dawley female rats to generate F1 heterologous transgenic line founders. Because of the crossing-over and meiotic reduction division, the number of genetically different sperm cells from single modified spermatogonia depends on a number of integrations (N) and equals 2N x 2. The identification of transgenic animals by traditional genotyping approach must be complemented with an assessment of the transgene activity. Taking into consideration a possibility of obtaining a wide variety of the offspring's transgene number and genomic localization we decided to perform initial animal screen based on an estimation of functional activity of ERT2CreERT2 in germ cells (Fig 2, B) and then promising candidates analyze more vigorously.

The first litter was born after seven months post-transplantation. Due to relative abundance of male germ stem cells and higher activity of DAZL promoter in spermatogonia, only male offspring were analyzed for a presence of functionally active ERT2CreERT2 transgene. From seven liters we obtained 35 male pups. In the course of survival surgery, left testes was removed from each animal while right testes remained undisturbed to preserve reproductive potential of prospective breeders. Each isolated testicle was used for individual isolation of laminin-binding (LB+) fraction from germ cell primary cultures according to an established protocol **(Chapman et al., 2011).** Harvested LB+ germ cells population was electroporated with reporter plasmid pCALNL-GFP using Neon transfection system (Life Technology). One part of the transfected cells was treated with a dose of 4-hydroxytamoxifen during one feeding in an attempt to activate the potentially present ERT2CreERT2 protein that would subsequently mediate removal of the Lox-Neo-Lox Stop cassette from the reporter plasmid and result in an observable expression of EGFP protein in live cells.

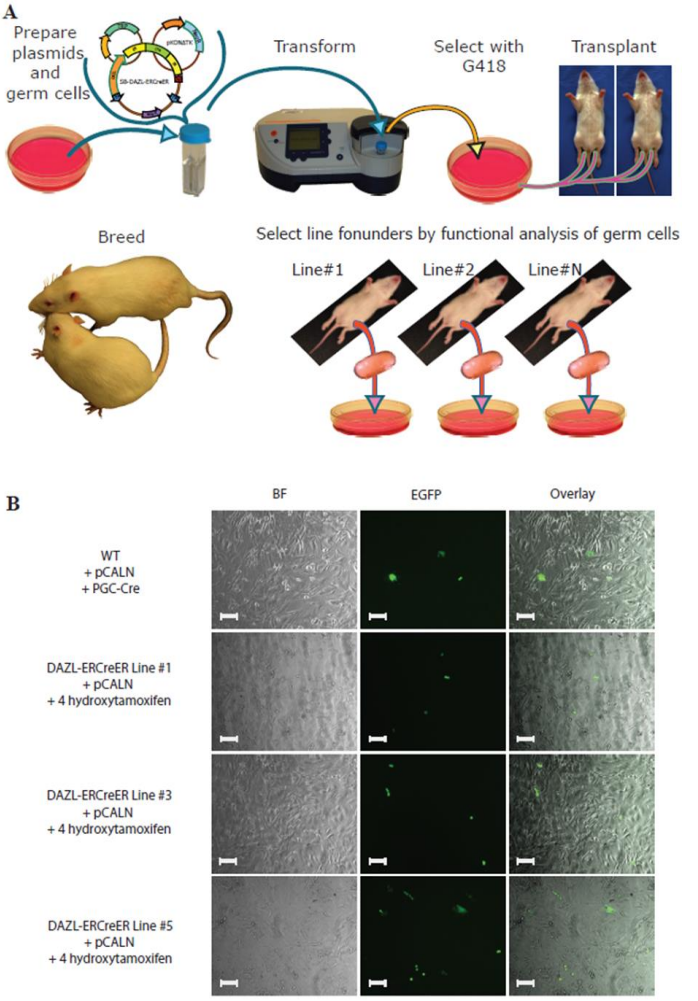

Figure 2 Generation and functional analysis of DAZL-ERT2CreERT2 transgenic rat line founders. **A**. A diagram of the workflow for generation and identification of F1 transgenic line founders; **B**. Functional analysis of LB^{+} spermatogonia isolated from a left testicle removed individually from the prospective F1 transgenic line founders. Cells were electroporated with pCALNL-GFP reporter plasmid (Addgene plasmid 13770) and treated with 4hydroxytamoxifen. Scale bar=100 μm

Using the described approach 23 males were identified as ERT2CreERT2positive with a functionally active ligand-inducible recombinant ERT2CreERT2 protein. Although somatic cells were present in the electroporated fraction, remarkably some lines had only spermatogonia expressing the reporter EGFP marker while others had EGFP expression in the somatic testicular cells (Fig 2, B). Totally six transgenic male pups were selected as lines founders. Line #1 was determined to contain a single integration of a transgene selectively expressed in germ cells. Comprehensive characterization of these lines will be presented elsewhere.

To test the functionality of DAZL-ERT2CreERT2 in germ cells we developed Sleeping Beauty constructs containing Cre excisable Lox-Neomycin-Lox stop cassette followed by fluorescent proteins or gene of interest. Using elements of the SB-DAZL parental vector and parts of the pCALN reporter vector we constructed two Sleeping Beauty based reporter plasmids: SB-DZeCAG-LoxNeoLox-EGFP, SB-DZeCAG-LoxNeoLox-TOMATO. It was expected that, after the addition of Tamoxifen or 4-hydroxytamoxifen, ligand-activated ERT2CreERT2 would mediate an excision of floxed Neomycin stop cassette thus activating expression of the reporter transgene. All constructs were pretested in the COS cell in a combination with the PGK-Cre (positive control) and SB-DAZL-ERT2CreERT2.

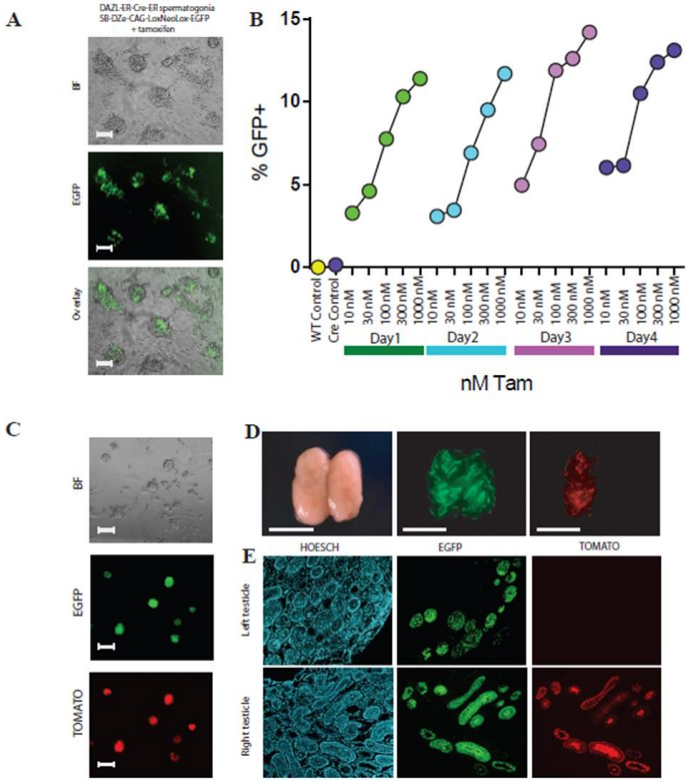

Figure 3 Analysis of the double and triple reporter transgenic cell lines
A. *In vitro* 4-hydroxytamoxifen induced, ERT2CreERT2 mediated activation of the expression of EGFP protein in the culture of DAZL-ERT2CreERT2 line #1 spermatogonia transformed with SB-DZe-CAGLoxNeoLox-EGFP reporter vector; **B.** Time-dose response of the DAZL-ERT2CreERT2 line #1 spermatogonia transformed with SB-DZe-CAG-LoxNeoLox-EGFP to 4-hydroxytamoxifen treatment estimated by the flow-sort analysis; **C.** Bright field, green and red fluorescence images of live cells of *in vitro* 4hydroxytamoxifen induced, ERT2CreERT2 mediated activation of the expression of TOMATO protein in the DAZL-ERT2CreERT2 /GCS-EGFP/DZe-CAG-LoxNeoLox-TOMATO spermatogonial cell line; **D.** Bright field, green and red fluorescence images of testes from WT recipient rats at 60 days post-transplantation. Testis transplanted with 150×10^3 of DAZL-ERT2CreERT2 /GCS-EGFP/DZen-CAG LoxNeoLox-TOMATO spermatogonial cell line with (Left) and without (Right) *in vitro* activated TOMATO reporter construct. Scale bar=1 cm.; **E.** Histological cross-sections of seminiferous tubules of the respected testes.

While the pCALNL-GFP plasmid had some leaky expression in the COS cells, the Sleeping Beauty based vectors demonstrated very tight control over the expression of the recombination cassette and no detectable expression of the transgenes in transfected cells were observed with a fluorescent microscopy. An untransfected control fraction of the cells isolated for functional analysis from the DAZL-ERT2CreERT2 male founder of line #1 was further expanded for seven passages. These cells were used to develop several clonally derived double transgenic cell lines utilizing the Sleeping Beauty based reporter construct SB-DZe-CAG-LoxNeoLox-EGFP, as well as construct designed to interrupt function of endogenous ERBB2 protein: SB-DZeCAG-LoxNeoLox-DNErbB2-V5. DAZL-ERT2CreERT2 cells were electroporated using the NEON system with corresponding vectors and Sleeping Beauty transposase plasmid in the same ratio used to conduct initial T138 cells modification with SB-DAZL-ERT2CreERT2.
Presence of a functional Neomycin Stop cassette floxed by Lox cites in the constructs provided a direct G418 selection of transfected cells. From 100 to 300 individual colonies of G418b selected spermatogonia were manually picked for each transfection. After the colony isolation, remaining total cell library modified with DAZL-ERT2CreERT2/SB-DZe-CAG-LoxNeoLox-EGFP (Fig 3, A) was used to test the time-dose response of germ cell to treatment with 4-hydroxytamoxifen *in vitro*. Flow-sort analysis revealed that a single treatment with 10 nM of 4-hydroxytamoxifen is sufficient for Cre mediated excision LoxNeoLox stop cassette and activation of GFP expression (Fig 3, B).
After initial expansion clonal cultures were divided into two parts. While one part served for line derivation, the second part was tested for transgene expression induced by Cre mediated recombination activated by 4-hydroxytamoxifen. On average 3-5% of picked clones demonstrated the effectiveness of tested ERT2CreERT2/LoxP system at a level detectable by the ICC and the fluorescent microscopy.
Additionally, DAZL-ERT2CreERT2 male from the line#1 was mated to a GCS-EGFP female to generate a double transgenic GCS-EGFP/DAZL-ERT2CreERT2 rat line. Since GCS-EGFP rat expresses green fluorescent protein strictly in germ cells we reasoned that such cross would be especially useful for studying spermatogenesis as it allows a direct *in vitro* observation and comparison of live cells with and without 4-tamoxifen activated Cre mediated genetic rearrangements. A freshly prepared culture of double transgenic germ cells was immediately tested for the functionality of ERT2CreERT2. A small fraction of the total germ cells mixture containing different types of cells was transfected with pretested DZe-CAG-LoxNeoLox-Tomato one more time demonstrating expression of ERT2CreERT2 selectively in the spermatogonia. The spermatogonial cell line was derived from this double transgenic rat to generate triple transgenic reporter cell line using SB-DAZLenCAG-LoxNeoLox-Tomato (Fig 3, C). Generation of this line was performed as described above. Derived lines were used to test *in vitro* and *in vivo* performance of the recombination system. For this purpose, cells were transplanted into busulfan treated WT Sprague Dawley recipients. Three weeks post-transplantation animals were administrated with filter sterilized sunflower oil based solution of 4-hydroxytamoxifen at 60 mg/kg. After three weeks post-treatment testis transplanted with experimental cells were analyzed. The analysis confirmed *in vivo* inducible Cre mediated recombination and activation of the expression of the TOMATO transgene (Fig 3, E; D).
Another approach was to induce transgenic cells *in vitro*. This tactic enables one to verify the event of recombination and estimate the number of positive cells prior to transplantation and, if necessary, select a positive clone for the line derivation.
Such strategy is permissible whenever planned genetic alteration is not expected to interfere with spermatogenesis if the goal is to obtain an animal with Cre mediated genomic alteration. In our case, this strategy was applied to DAZL-ERT2CreERT2/DZe-CAG-LoxNeoLox-TOMATO spermatogonial cell line. Cells were treated with 4-hydroxytamoxifen during one feeding to activate an expression of TOMATO protein. The rate of recombination based on green vs. green-red cell count was estimated to be not lower than 75% in the population of cell harvested for the transplantation. Activated and not activated cells were transplanted into different testis of the same animal. Testes were removed after 60 days post-transplantation and analyzed microscopically (Fig 3, D). The number of tubules expressing GFP and TOMATO fairly reflected the estimated rate of *in vitro* recombination. Microscopic analysis of testicles cross-sections revealed both none activated – green cells and activated – green/red cells at different stages of the development including maturating sperm (Fig 3, E). An attempt to generate transgenic animals using described cell lines is underway.

CONCLUSION

In this work we demonstrated a successful production of custom designed transgenic rat model via modification of spermatogonial stem cell *in vitro* followed by transplantation of modified cells into recipient animal *in vivo*. Obtained rat demonstrated tightly regulated and inducible expression of ERT2CreERT2 transgene in the reproductive cell line.
Thus, generated DAZL-ERT2CreERT2 animal and its germ cells can be effectively utilized to study spermatogenesis in a rat **(Syvyk et al., 2017).** Additionally, this animal can potentially be bred to animals caring floxed DNA for the germline activation and transmission of designed genomic alterations if such alterations will not interfere with the spermatogenesis. Moreover, since the sequence of the ERT2CreERT2 fusion protein is flanked by attR sites, theoretically it could be replaced with any sequence of interest flanked by attL sites providing that cells are supplemented with LR recombinase enzyme complex and a donor sequence.

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