ARTICLE INFO

ABSTRACT

Spermatogenesis mediated transgenesis is becoming an increasingly popular method of genetic modification in animals. It guarantees direct germline transmission because spermatogenic stem cells are the unipotent type of stem cell and can differentiate only into the mature spermatocytes. Additionally, modified spermatogenic 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. In obtained animal tamoxifen inducible engineered ERT2CreERT2 recombinase is specifically expressed in germ cells at a level sufficient for ligand-dependent efficient recombination. Using male germ cells from a generated animal and its cross with GCS mice, we demonstrated that Cre-dependent transformation of spermatogonial stem cells generated by gene targeting in vitro 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 allowed 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 genetic modification of timely 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.

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 species. 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 519 bp 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 atR 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 – CACCCGTCGACCCATGCAATCTACTG; Reverse primer – AGCTTGTTGCAAGGAAAACCTCCTG). 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 pNeoO TK vector was prepared from pNeoO TK (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 Olsson laboratory and was used as positive control in test transfections and during functional analysis. pSB-DAZL-EGFP 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-DAZL-CAG-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. The PCR product of ERT2CreERT2 fusion protein was amplified from Addgene plasmid 13777 and cloned into pENTR/D-Topo containing 3.6 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 pNeoO TK 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. After 24 hours, one well with SB-DAZL-ERT2CreERT2 transfected cell were supplemented with medium containing 1µM of 4-hydroxytamoxifen. 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.

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 was 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 pCALNL-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. 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 laminating 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 pNeoO TK 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. After 24 hours, one well with SB-DAZL-ERT2CreERT2 transfected cell were supplemented with medium containing 1µM of 4-hydroxytamoxifen.

Generation of chimeric Recipient-Producer 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 congenic progeny from genetically modified spermatogonia was obtained. The PCR product of ERT2CreERT2 fusion protein was amplified from Addgene plasmid # 14797 and cloned into pENTR/D-Topo containing 3.6 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 pNeoO TK 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. After 24 hours, one well with SB-DAZL-ERT2CreERT2 transfected cell were supplemented with medium containing 1µM of 4-hydroxytamoxifen, the dose of 100µM 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 superstar.

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.
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 pKONATK 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 were mated with WT Sprague Dawley female rats to generate F1 transgenic line founders. Cells were electroporated with pcALNL-GFP reporter plasmid (Addgene plasmid 13770) and treated with 4-hydroxytamoxifen. 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 spermatogonial 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 pcALNL reporter vector we constructed two Sleeping Beauty based reporter plasmids: SB-DZeCAG-LoxNeoLox-EFGP, 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.
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-hydroxytamoxifen activated Cre mediated genetic rearrangements. A freshly prepared culture of double transgenic germ cells was immediately cultured 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-DAZL-CAG-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 administered with filter sterilized sunflower oil based solution of 4-hydroxytamoxifen at 60 mg/kg. After three weeks post-treatment tests 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 tests 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 maturing sperm (Fig 3, E, A).

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 (Svyyk et al., 2017). Additionally, this animal can potentially be bred to animals carrying 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 transgene is flanked by attL sites, theoretically it could be replaced with any sequence of interest flanked by attL sites providing that cells are supplemented with LR recombine enzyme complex and a donor sequence.

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