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All-in-One inducible lentiviral vector systems based on drug controlled FLP recombinase

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ABSTRACT

Site specific recombinases are frequently used as gene switches in transgenic animals where recombination is induced by drug treatment or by tissue specific recombinase expression. Alternatively, lentiviral gene transfer can be utilized for the genetic modification of a wide variety of cell types, albeit systems for tight control of transcriptional activity are scarce. Here, we combined lentiviral gene transfer and the development of a tightly drug-controlled FLP recombinase for the construction of "All-in-One" inducible gene expression systems. Tight control of FLP activity was achieved through N-terminal fusion with a FKBP12-derived conditional destruction domain and a C-terminal estrogen receptor binding domain making recombination dependent on the presence of Shield−1 and 4-hydroxytamoxifen. Exploiting the capacity of FLP to mediate excision and inversion, "All-in-One" lentiviral gene switch vector systems were generated where drug-induced recombination resulted in abrogation of FLP expression and subsequent overexpression or knockdown of the prototypical tumor suppressor phosphatase and tensin homolog PTEN. "All-in-One" vectors proved their functionality in a variety of hematopoietic cell lines, and primary murine bone marrow cells. Our new vector system thus combines the ease of lentiviral gene transfer and the power of site specific recombinases for analysis of gene function.

1. Introduction

Site specific recombinases (SSRs) are powerful tools for altering genome composition. The *S. cerevisiae* derived FLP recombinase targets a 48 bp FLP recognition target (FRT) site. FRTs consist of three 13 bp long FLP binding repeats that surround a central asymmetric 8 bp spacer that grants directionality. FLP excises DNA when two FRT sites share the same orientation, or mediates inversion of DNA segments flanked by FRT sites with opposing orientation [1]. Native FLP functions optimally at 30 °C and is therefore relatively inefficient in mammalian cells [2]. Stepwise optimization of its thermal stability (FLPe), nuclear import, and codon-usage (FLPo & hFlpe) resulted in FLP mutants suitable for highly efficient genetic modification of murine and human cells [3–7].

In reverse genetic screens, SSRs such as CRE or FLP, are often utilized as conditional gene switches to permanently initiate or abrogate gene expression [1]. Enormous research activities have been directed towards the generation of conditional mouse strains and murine embryonic stem cell lines with the aim to target every protein coding gene [8]. To gain spatiotemporal control over recombinase activity *in vivo*, expression from inducible or cell-type specific promoters can be utilized [1]. Additionally, SSRs fused to a mutated human estrogen receptor ligand binding domain (ERT2) have been employed [9–12]. In this system, Hsp90 sequesters fusion proteins in the cytoplasm but allows for nuclear translocation of the recombinase upon treatment with inducing drugs, e.g. the clinically applicable estrogen antagonist tamoxifen and its active metabolite 4-hydroxytamoxifen (4-OHT) [11]. Unfortunately, tamoxifen inducible SSRs are prone to leaky activation, which might result in unwanted phenotypes and cytotoxicity [13–16].

An alternative system for control of protein activity utilizes conditional destabilization domains [17–19]. For example, the FK506- and rapamycin-binding protein FKBP12 F36V/L106P double

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mutant (FKBP12) causes degradation of fusion proteins in the absence of the stabilizing ligand Shield-1 [18]. Degradation efficiency depends on FKBP12 N- or C-terminal fusion, the subcellular localization of the fusion protein, and the intrinsic properties of the protein of interest [18,20]. Like ERT2, FKBP12-mediated regulation occurs at the post-transcriptional level, and is therefore fast and fully reversible [18,20,21].

To perform functional studies independent of the generation of transgenic animals, retroviral vectors are suitable tools for constitutive or conditional overexpression or knockdown of genes [22–27]. The most frequently used inducible vectors contain tetracycline regulated promoters, which can be challenging to use due to a network of factors, such as the expression level of the transactivator, chromosomal position effects and doxycycline concentration, each of which may influence expression characteristics [28].

In order to combine the ease of retroviral gene transfer and the power of spatiotemporal controlled gene switches, we opted to develop a tightly regulated human codon-optimized FLP mutant (arbitrarily named FLPs) expressed from a lentiviral vector. Through fusion of FLPs to an N-terminal FKBP12 conditional destruction domain and a C-terminal ERT2 domain, recombination should be induced by Shield-1 and 4-OHT treatment. We tested the functionality of the new vectors for induced recombination *in trans* (in cells harboring FLP target loci) and *in cis* (with vectors containing the recombinase and the target allele) in fibroblasts and hematopoietic cells, and evaluated the potency of the vectors for overexpression and knockdown of proteins.

2. Material and methods

2.1. Cloning of constructs

Murine codon-optimized FLP recombinase (FLPo [6]) was used as a template for the generation of a human codon-optimized FLP version (FLPs) flanked by a 5′ FKBP12 F36V/L106P double mutant for conditional destruction [18] and a 3′ ERT2 domain for (4-hydroxy-) tamoxifen controlled activity [11]. Human codon-optimization of FKBP12-HAnlsFLPs-ERT2 was first performed using the GeneArt algorithm and then corrected for cryptic splice sites using the “FruitFly.org” splice site prediction algorithm. Gene synthesis was performed by Genscript (Piscataway, NJ, USA). The pUC57-FKBP12-HAnlsFLPs-ERT2 plasmid was subsequently used to generate all permutations of the parental construct by using (mainly) unique restriction sites between the different modules. Cloning details are available on request.

2.2. Cell culture and virus production

293T, SC-1 and SC-1 FlpR cells were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose; Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin and 0.1 mg/ml sodium pyruvate (all PAA, Pasching, Austria). 32D cells were cultivated in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin (all PAA), and 2 ng/ml murine IL3 (Peprotech, Hamburg, Germany). K562 cells were cultured in the same medium without IL3. Primary murine bone marrow (BM) cells stably expressing HoxA9 were cultured in DMEM supplemented with 15% FBS, 100 U/ml penicillin/streptomycin, 6 ng/ml mIL3, 10 ng/ml hIL6 and 20 ng/ml mSCF.

Viral particles were produced in 293T cells with standard $CaPO_4$ or polyethylenimine assisted transfection according to standard procedures. Per 10 cm plate, 5–7 μg lentiviral vector, 12 μg lentiviral gag/pol, 6 μg Rev, 1.5 μg VSVg or 2 μg ecotropic envelope were used. In some cases, pAdvantage (Promega, Fitchburg, WI, USA) plasmid was added to the transfection mix to increase lentiviral titers. Cells were washed 12 h after transfection, and viral supernatants were collected 24–36 h later. Virus supernatants were either directly frozen at −80 °C for later use or were subjected to ultracentrifugation at 10,000 rpm overnight or at 25,000 rpm for 2 h at 4 °C. Resuspended virus pellets were then stored at −80 °C for later use.

For transduction of SC-1 and SC-1 FlpR cells, 7x10e4 cells were seeded the day before transduction in a single well of a 12-well-plate. The following day, DMEM medium was replaced and supplemented with 4 μg/ml protamine sulfate (Sigma, Seelze, Germany) before addition of viral supernatants. Spin oculation occurred at 2,000 rpm at 32 °C for 1 h. 2x10e5 32D and K562 cells were transduced per well of a 24 well-plate in protamine sulfate containing medium by spin oculation. HoxA9 bone marrow cells were transduced at a density of 1x10e5 cells per well in 96-well-plates by spin oculation using media containing protamine sulfate.

2.3. Transplantation of HoxA9 BM cells

Animal experiments were approved by the supervising animal research review board at Hannover Medical School, and mice were kept in specific pathogen-free conditions in the animal facility of Hannover Medical School. 1x10e6 *in vitro* expanded C57BL/6 Ly5.2 derived HoxA9 BM cells were transplanted into sublethally (8 Gy) irradiated female C57BL/6 Ly5.1 mice in combination with 1x10e5 fresh Ly5.1 BM cells. Mice were given ciprofloxacin (Fresenius Kabi Deutschland, Bad Homburg, Germany) for the first three weeks after transplantation, and were monitored on a regular basis.

2.4. Reagents

Shield-1 (Cheminpharma LLC, Farmington, CT, USA) and 4-OHT (Sigma-Aldrich, Seelze, Germany) were resuspended in ethanol to generate 1 mM stocks (1000×) used for cell culture experiments. Transduced cells were treated with 1 μM Shield-1, 1 μM 4-OHT or a combination of both, while control cells were either incubated with 1% or 2% (v/v) EtOH, or left untreated. When groups with 4-OHT and 4-OHT/Shield-1 were analyzed in parallel, control cells were treated with 2% (v/v) EtOH. Treatment occurred for seven days, unless stated otherwise. Cells were split and seeded with fresh medium containing supplements when confluence was reached.

2.5. RT-QPCR

Fresh or frozen cell pellets were subjected to RNA extraction using RNAzolRT (Molecular Research Center INC., Cincinnati, Ohio, USA) solution according to the manufacturer's instruction. One μg of RNA was used for cDNA synthesis using Quantitec Reverse Transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For RT-qPCR, cDNA from untransduced cells was used in four 10-fold dilutions to generate a standard curve for primer efficiency calculation and subsequent determination of target gene expression in transduced cells by the Pfaffl method [29]. The following primers were used in this study: murine PTEN FW 5′-tgg aat cga ctt aga ctt gac ct-3′, murine PTEN RV 5′-gcg gtg tca taa tgt ctc tca g-3′, murine/human beta-Actin FW 5′-cct ccc tgg aga aga gct a-3′, murine beta-Actin RV 5′-tcc ata ccc aag aag gaa gg-3′, human beta-Actin RV 5′-tcc atg ccc agg aag gaa g-3′; human PTEN FW 5′-gtt tac cgg cag cat caa at-3′, human PTEN RV 5′-ccc cca ctt tag tgc aca gt-3′. PTEN qPCR primers have been published before [30]. Quantitative PCRs were performed on the ABI StepOne Plus (Applied Biosystems, Darmstadt, Germany).

2.6. Vector copy number determination

Vector copy number determination by *TaqMan* quantitative PCR (ABI Taqman Fast Advanced Master Mix; Applied Biosystems) was perfomed on genomic DNA samples purified with the QIAamp DNA Blood Mini Kit (Qiagen) using vector specific wPRE primers and probe (PRE FW 5′-gag gag ttg tgg ccc gtt gt-3′ and PRE RV 5′-tga cag gtg gtg gca atg cc-3′; 5′FAM-ctg tgt ttg ctg acg caa c-BHQ1-3′) in combination with PTBP2 specific primers and probe (PTBP2 FW 5′-tct cca ttc cct atg ttc atg c-3′ and PTBP2 RV 5′-gtt ccc gca gaa tgg tga ggt g-3′; 5′JOE-atg ttc ctc gga cca act tg-BHQ1-3′) for normalization of input DNA. Vector copy numbers were calculated based on the efficiency of the PCR reaction determined by serial dilutions of a “4xStandard” plasmid containing PCR target sequences for eGFP, wPRE, Flk-1 and PTBP2, and subsequent copy number calculation according to Pfaffl [29]. *TaqMan* quantitative PCRs were performed on the ABI StepOne Plus (Applied Biosystems) in triplicates.

2.7. PCR for molecular detection of recombination events

Detection of recombination events was performed by standard PCR using *Taq* DNA polymerase (Qiagen) in combination with its standard buffer and 0.5× “combinational enhancer solution” (CES) on 200 ng of genomic DNA purified with the QIAamp DNA Blood Mini Kit (Qiagen) [31]. For detection of excision events in K562 cells transduced with GFP and GFP-PTEN inducible vectors, PCRs were performed with three primers SFFV FW 5′-acc aat cag cct gct tct cgc t-3′, PuroR FW 5′-cag cct gct gaa gca ggc cgg cga cgt gga gga gaa ccc cgg tcc ggt ga-3′ and GFP RV 5′-gga ctg ggt gct cag gta gtg g-3′, yielding products of 1343 bp (full-length) or 798 bp (excised). For detection of transgene inversion in cells transduced with inducible shRNA constructs, a three primer mix SFFV FW 5′-acc aat cag cct gct tct cgc t-3′, GFP FW 5′-cta tat cat ggc cga caa gca ga-3′ and GFP RV 5′-gga ctg ggt gct cag gta gtg g-3′ yielded products of 165 bp (parental configuration) or 798 bp (inversed configuration). Untreated cells (MOCK) and water (H_2O) were used as control. Selected PCR products were subcloned and sequenced to verify recombination events and PCR product identity.

2.8. Western blot

Western blot analysis was performed according to standard protocols [27] using 10–25 μg of total cellular proteins with antibodies α-HA-Tag (clone 6E2; mAb #2367; Cell Signaling Technology, Danvers, MA, USA, and α-histone H2B (ab1790, abcam, Cambridge, UK)).