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Short report

Improved transgene expression in doxycycline-inducible embryonic stem cells by repeated chemical selection or cell sorting

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ABSTRACT

Transgene-mediated programming is a preeminent strategy to direct cellular identity. To facilitate cell fate switching, lineage regulating genes must be efficiently and uniformly induced. However, gene expression is often heterogeneous in transgenic systems. Consistent with this notion, a non-uniform reporter gene expression was detected in our doxycycline (DOX)-regulated, murine embryonic stem (ES) cell clones. Interestingly, a significant fraction of cells within each clone failed to produce any reporter signals upon DOX treatment. We found that the majority of these non-responsive cells neither carry reporter transgene nor geneticin/G418 resistance. This observation suggested that our ES cell clones contained non-recombined cells that survived the G418 selection which was carried out during the establishment of these clones. We successfully eliminated most of these corrupted cells with repeated chemical (G418) selection, however, even after prolonged G418 treatments, a few cells remained non-responsive due to epigenetic silencing. We found that cell sorting has been the most efficient approach to select those cells which can uniformly and stably induce the integrated transgene in this ES cell based platform. Together, our data revealed that post-cloning chemical re-selection or cell sorting strongly facilitate the production of ES cell lines with a uniform transgene induction capacity.

1. Introduction

Transgene-mediated cell conversion is a widely used strategy to manipulate cellular identity (Graf and Enver, 2009). Ectopically expressed transcription factors are routinely used to reprogram somatic cells into induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Yamanaka, 2012), moreover overexpression of lineage determining transcription factors can stimulate direct lineage conversions (Chambers and Studer, 2011; Lee and Young, 2013; Morris and Daley, 2013). In addition, embryonic stem (ES) cell differentiation can be directed by forced expression of lineage restricted transcription factors (Kyba et al., 2002; Wang et al., 2005). To achieve a controllable, uniform expression, transgenes are often integrated into a specific chromosomal locus and their expression is driven by a DOX-responsive promoter. Numerous technologies are available for targeting a gene of interest into a predetermined genomic region. For example, homologous recombination can be employed using genome editing technologies (Merkert and Martin, 2016), or recombination mediated cassette exchange can be carried out on a pre-modified genomic locus (Ting et al., 2005; Turan et al., 2013). In this report we applied a recently developed inducible cassette exchange recombination method (Iacovino et al., 2011; Iacovino et al., 2014) for site specific gene targeting. We used a mouse ES cell line (ZX1) (Dandapat et al., 2014), that encodes a DOX-inducible floxed Cre, which substitutes itself with an incoming gene of interest upon recombination (Iacovino et al., 2011; Iacovino et al., 2014). In parallel, a special promoter plus ATG sequences are inserted upstream of the neomycin resistance gene which restores resistance to neomycin/geneticin in the recombined ES cells (Fig. 1). Various versions of this inducible system have been applied to enhance hematopoietic or skeletal muscle development by forced expression of lineage specific transcription factors (Darabi et al., 2008; Kyba et al., 2002).

In spite of these advanced gene targeting technologies, there is a general problem with the transgenic systems, namely heterogeneity and instability of transgene expression. Consistent with this notion, in this report, we observed a heterogeneous transgene induction in our DOX-regulated ES cell clones. We found that the majority of the non-

Abbreviations: azadC, 5-aza-2′-deoxycytidine; DOX, doxycycline; EGFP, enhanced green fluorescent protein; ES cell, embryonic stem cell; ICE, inducible cassette exchange.

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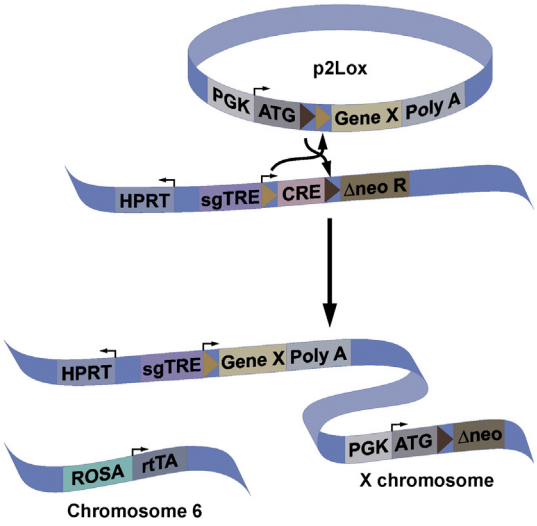

Fig. 1. Inducible cassette exchange recombination. The targeting plasmid (p2lox) can be recombined with the inducible cassette exchange (ICE) target locus, which is located upstream of the Hprt gene on the X chromosome. At the ICE target locus a second generation tetracycline response element (sgTRE) drives expression of a Cre transgene before recombination. Arrows indicate recombination between homologous loxP sites that are represented by triangles. After Cre-mediated recombination the incoming gene is placed downstream of the sgTRE, and the PGK-promoter plus ATG sequences are introduced upstream of the neo (neomycin/geneticin resistant) gene. In addition, ZX1 ES cells constitutively express the reverse tetracycline transactivator (rtTA), which is encoded in the ROSA26 locus (on Chromosome 6).

responsive ES cells did not carry reporter transgene suggesting that non-recombined cells survived/reformed during the establishment of these cell lines. Importantly, these non-responsive cells can be efficiently eliminated by chemical (G418) re-selection or cell sorting.

2. Materials and methods

2.1. Chemicals

Na-butyrate, 5-aza-2′-deoxycytidine (azadC) and doxycycline (DOX) were obtained from Sigma-Aldrich. Geneticin (G418) was purchased from Life Technologies.

2.2. Targeting vector construction

The coding sequences of the EGFP and mCherry genes were amplified with Gateway compatible attB-attached primers. Primer sequences are shown in Table S1. The PCR products were subcloned into the pDONR221 entry plasmid with Gateway technology (Invitrogen-Life Technologies). The entry clones carrying the transgenes were recombined with a Gateway compatible plasmid vector (p2lox-GW).

2.3. ES cell culture

The parental ZX1 mouse ES cell line (Dandapat et al., 2014) and the newly generated mouse ES cell clones were cultured on primary mouse embryonic fibroblast (MEF) feeder layers during the establishment and expansion of the genetically modified ES cell lines. In contrast, ES cells were maintained without MEF feeder on gelatin pre-coated dish for testing the transgene inducibility. ES cells were maintained in knockout DMEM (Life Technologies) as described (Szatmari et al., 2010).

2.4. Generation of DOX-inducible ES cell clones

DOX-inducible ES cell clones were produced as described (Iacovino et al., 2014) with minor modifications. In brief, ZX1 ES cells were treated with DOX (1 μg/ml) one day before transfection in order to induce the Cre enzyme. The transgene bearing p2lox targeting vectors were introduced into the ZX1 cells by electroporation using the Neon Transfection System (Life-Technologies). For electroporation single pulse was applied with 1400 V at 10 ms. After electroporation ES cells were seeded on G418 resistant, mitomycin-C treated, mouse embryonic fibroblast (PMEF-N; Merck-Millipore) layers in 6-well plates and selected with 300 μg/ml G418 containing knockout-DMEM medium (Life-Technologies) for 7–8 days. After selection ES cell colonies were picked and replated on MEF layers for expansion.

2.5. Genomic PCR

Genomic DNA was purified with a High Pure PCR Template Preparation Kit (Roche). Quantitative PCR was carried out by real-time PCR (Light Cycler 480; Roche) as described (Szatmari et al., 2010). In brief, 40 cycles at 95 °C for 12 s and 60 °C for 30 s using fluorescence reporter probes. Assay ID or primer/probe sequences are listed in Table S1. The mCherry specific PCR reactions were done in triplicate, relative copy number was determined by the comparative threshold cycle method and normalized to the Maf gene.

Cre gene was detected with end-point PCR using a Cre-specific primer set (primer sequences are in Table S1). 100 ng genomic DNA was amplified under the following conditions: 3 min at 94 °C; 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30; and a final extension at 72 °C for 5 min.

2.6. Flow cytometric analysis and cell sorting

Flow cytometric analysis and cell sorting was performed with a FACS Aria III (BD Biosciences) sorter. 488 and 561 nm lasers were used to excite EGFP and mCherry, respectively and fluorescence signals were collected with a 530/30 band pass (BP) filter for EGFP and 610/20 BP filters for mCherry. For cell separation approximately150,000 mCherry positive and negative ES cells were sorted. Sorted cells were expanded in knockout-DMEM medium using feeder-free ES cell culture conditions.

3. Results

3.1. Bimodal distribution of reporter signal in DOX-induced ES cells

Transgene-mediated programming is a popular strategy to manipulate cellular identity during ES cell differentiation. We intended to optimize this transgene mediated cell conversion approach, therefore we created inducible ES cell clones carrying reporter transgenes. DOX-controlled cell clones were engineered with a previously developed inducible cassette exchange recombination method (Iacovino et al., 2011; Iacovino et al., 2014) as described in Materials and methods. In this transgenic system, the properly recombined, individual male ES cells carry one copy of integrated DNA segment in chromosome X (Fig. 1). Therefore, this platform allows us to monitor the expression of a single copy, inducible transgene.

First, we examined the mCherry reporter gene expression of the individual ES cell clones with fluorescence microscopy. Strikingly, among the mCherry bright cells we observed numerous cells lacking any apparent reporter signals in all of the investigated ES cell clones (Fig. 2A). In the following experiments we quantitated the distribution of the reporter positive and negative cells in the whole population by flow cytometry. Consistent with the microscopic data, an overt intraclonal transgene expression heterogeneity was obtained in the reporter carrying ES cells. All of the tested ES clones exhibited a bimodal distribution of mCherry reporter signal (Fig. 2B and C). Bimodal distribution means that a fraction of cells failed to produce any reporter signals, in contrast the rest of the cells were mCherry positive. Similarly, heterogeneous reporter expression patterns were detected in EGFP carrying ES cell clones (Fig. S1) suggesting that the bimodal distribution profile is a general characteristic of this inducible system. In summary, our analyses