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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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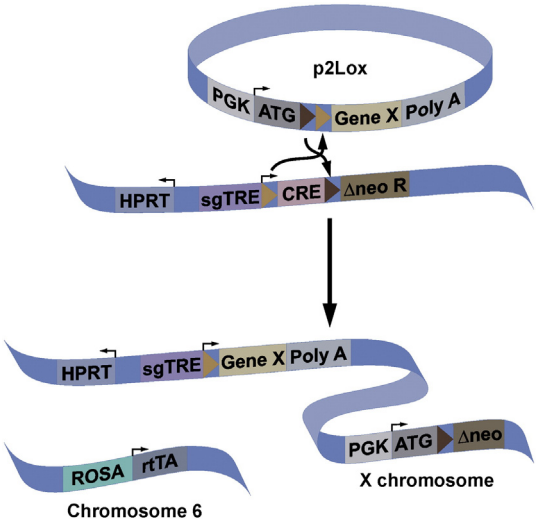

Fig. 1. Inducible cassette exchange recombination. The targeting plasmid (p2lox) can be recombined with the inducible cassette exchange (ICE) target locus, which is located upstream of the Hprt gene on the X chromosome. At the ICE target locus a second generation tetracycline response element (sgTRE) 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

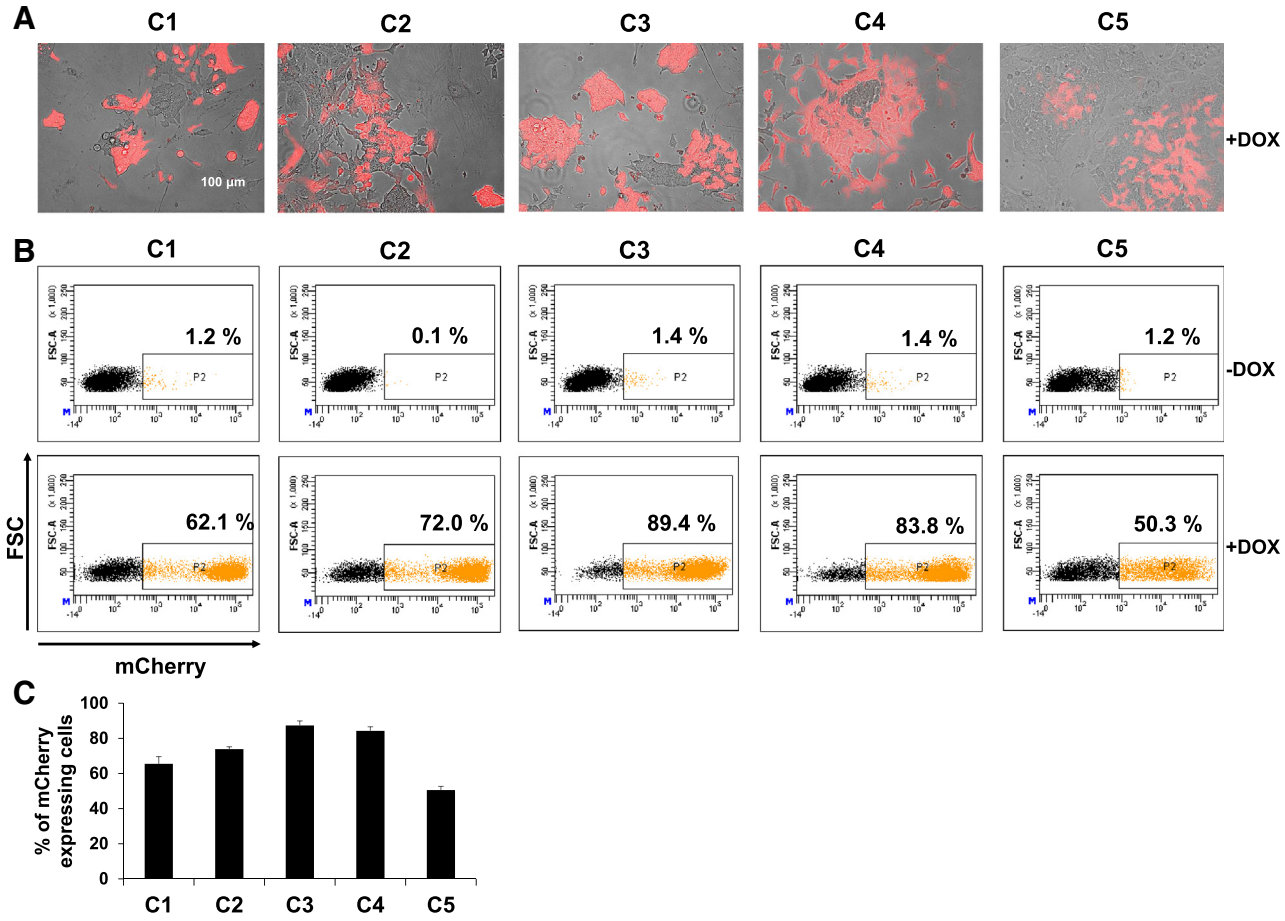

Fig. 2. Bimodal expression of mCherry protein in DOX treated ES cell clones. ES cell clones (C1–C5) were cultured in the presence (2 μg/ml) or absence of DOX for 2 days. (A) Microscopic detection of fluorescence reporter signals from mCherry carrying ES cell clones. Composite pictures (phase contrast plus fluorescence) were generated with a Floid cell imaging station (20× plan fluorite objective, scale bar is indicated). (B) Representative flow cytometric profiles of the mCherry specific clones. The mCherry fluorescence is plotted on the x axis; forward scatter (FSC) is plotted on the y axis. The percentage of cells falling within the positive gate is shown. (C) Average percentage of the mCherry positivity of the DOX-treated (2 μg/ml) reporter cell clones. The average and the SD values were calculated from 3 independent experiments.

revealed that, DOX-regulated ES cell clones exhibited a non-uniform reporter expression and a subset of ES cells was unable to express the transgene.

3.2. Improved transgene induction upon repeated chemical (G418) selection

Our flow cytometric analyses indicated that at least 10% of the cells failed to express any reporter signals in the investigated ES cell clones. It is possible that the non-responsive cells had insufficient sensitivity to DOX or a limited drug-uptake capacity. To test this possibility, two transgenic ES cell clones were treated with graded amounts of DOX (2–8 μg/ml) for two days (Fig. 3A). Despite of the elevated concentrations of DOX, we failed to obtain a consistently improved reporter gene expression, suggesting that antibiotic concentration is not the limiting factor in this inducible system.

To further investigate the potential reasons of the expression heterogeneity, we took the advantage that during the targeting plasmid integration a functional neomycin resistance gene is formed that renders the recombined ES cells resistant to neomycin/G418 (Fig. 1). We employed a prolonged (7–8 days) G418 (300 μg/ml) selection during the establishment of these ES cell clones to eliminate the non-recombined ES cells. Despite of this initial chemical selection, however, it is possible that some non-recombined ES cells survived or some of them lost their G418 resistance during the ES cell expansion which were carried out without chemical (G418) selection. To verify this possibility, the mCherry specific ES cell clones were cultured in the presence or absence of G418 (300 μg/ml) for 8 days. Interestingly, elevated percent of mCherry expressing cells was detected in all transgenic clones although we never reached 100% positivity after re-selection (Fig. 3B). This finding suggested that a considerable fraction of the non-responsive cells were G418-sensitive; elimination of these cells by a second round of G418 selection substantially improved the transgene inducibility of these ES cells.

3.3. Genetic heterogeneity is responsible for the impaired transgene induction

Our data revealed that a fraction of cells was refractory to DOX treatment in all investigated mCherry bearing ES cell clones and most of these unresponsive cells were G418 sensitive. We reasoned that either genetic alterations and/or epigenetic silencing were responsible for the compromised transgene expression. Therefore, we tested the integrity of the mCherry transgene from the DOX responsive and non-responsive ES cells. To achieve this, two mCherry specific cell clones (C2 and C4) were sorted with FACS. The separated cells were expanded for three days in the absence of DOX and re-stimulated with DOX for 48 h. 97–99% of the positive cells retained their inducibility, while most of the negative cells remained non-responsive in the presence of DOX (Fig. 4A). This finding confirms that these ES cells can stably maintain their transgene inducibility, even after several rounds of cell division. In addition, this result indicates that cell sorting is a very efficient

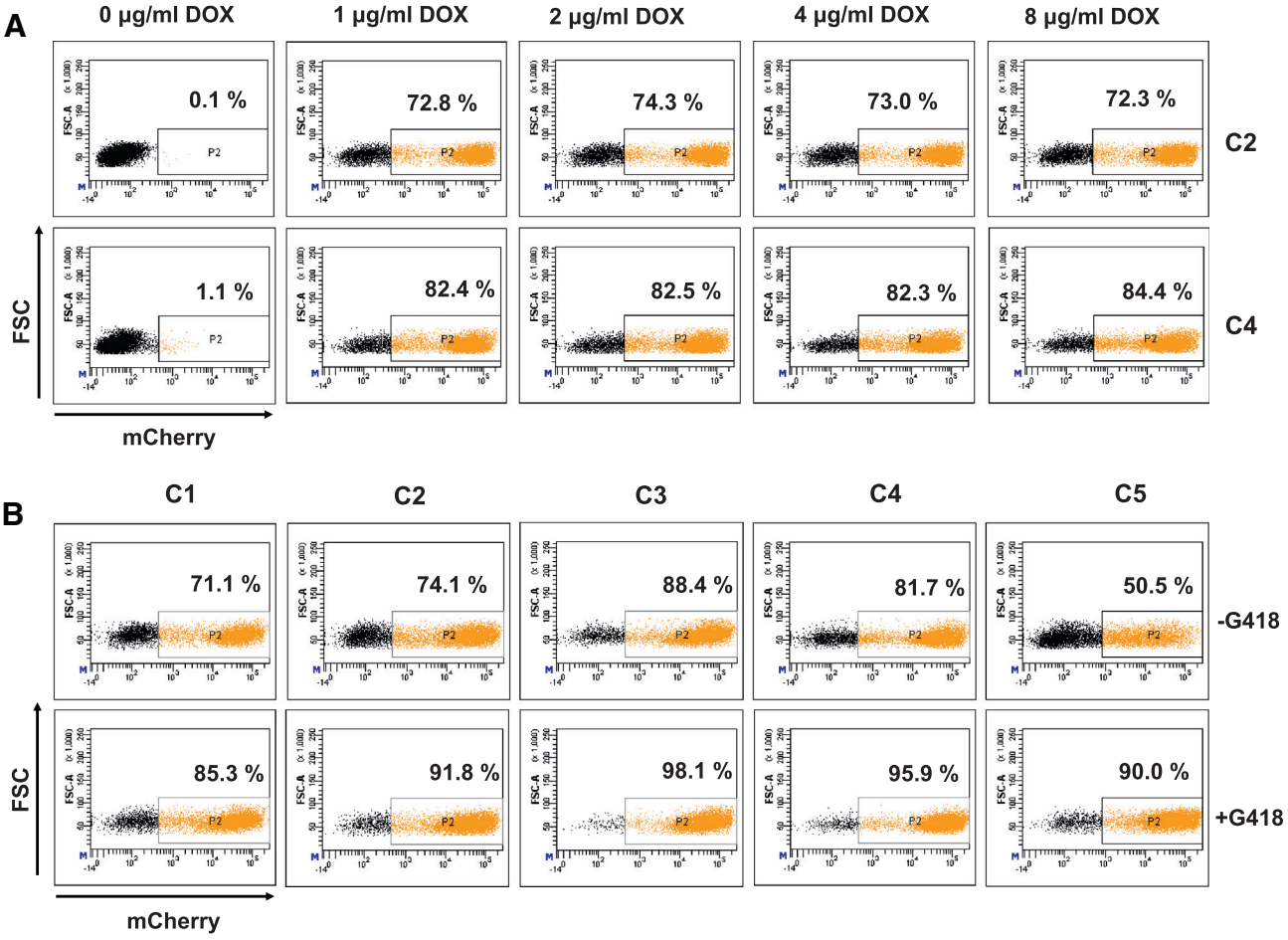

Fig. 3. Similar transgene induction with high concentration DOX treatment, but enhanced reporter gene expression after G418 re-selection. (A) The mCherry specific C2 and C4 ES cell clones were treated with the indicated concentrations of DOX for 2 days. (B) Flow cytometric profiles of the mCherry bearing clones (C1–5). ES cells were cultured for 8 days in the presence or absence of 300 µg/ml G418 and re-stimulated with DOX (1 µg/ml) for 2 days.

method to enrich those cells which uniformly express the integrated transgenes.

Next we determined the relative gene copy number of mCherry from the sorted ES cells. The mCherry copy number was measured with QPCR and normalized to an intronless endogenous gene (Maf). Approximately 0.4 mCherry/Maf ratios were detected from the sorted positive cells (Fig. 4B). This value is near to the 0.5 ratio which is expected if we consider that two copies of Maf gene can be found per cell, but only one copy of mCherry integrated into the single chromosome X, in these male ES cells. In striking contrast to the positive cells, however, mCherry gene was barely detected from the sorted negative cells (Fig. 4B), but the Cre specific PCR product was readily detected from these cells (Fig. 4C). Of note, in our genetically modified ES cells (ZX1) the Cre gene is supposed to excise itself upon recombination and should be replaced with the mCherry transgene (Fig. 1). These data suggest that a few G418 sensitive and reporter free ZX1 like ES cells remained in the ES cell clones. Consistent with this finding, the sorted negative cells exhibited a robust cell death upon G418 re-selection and the recovered cells showed a much stronger reporter gene induction (Fig. 4D). Moreover, these G418 re-selected ES cells had a higher mCherry/Maf ratio confirming that the G418 sensitivity and the reporter gene deficiency were tightly associated (Fig. 4E). Together, these results indicated that a subset of cells within each clone was reporter gene free and this population substantially attributed to the impaired inducibility of the transgene.

3.4. Epigenetic gene silencing can also contribute to the compromised transgene induction

Data shown above demonstrated that we could eliminate large part of the non-inducible cells with chemical re-selections, however, we failed to obtain 100% mCherry expression even after a prolonged G418 treatment (Fig. 3B). Moreover, 10–50% of the sorted, non-responsive, but G418 re-selected ES cells remained refractory to DOX treatment (Fig. 4D). These results imply that additional mechanisms may contribute to the impaired transgene induction. We assumed that DNA methylation and/or histone acetylation might participate in the repression of the reporter transgene. To test this possibility, G418 re-selected ES cells were treated with azadC (5-aza-2′-deoxycytidine) or Na-butyrate for 48 h. AzadC (Decitabine) is a well-known DNA hypomethylating agent (Lubbert, 2000). Na-butyrate is an inhibitor of histone deacetylases (Mariani et al., 2003). In line with the DNA methylation and histone deacetylation depending silencing, elevated percentage of mCherry positive cells was detected in the presence of azadC or Na-butyrate in the G418 re-selected cells (Fig. 5A and B). These findings suggest that DNA methylation and histone acetylation dependent processes are partly responsible for the transgene repression. However <2% mCherry positive cells were detected in the sorted samples, which were not re-selected with G418, in the presence of these inhibitors (Fig. 5C). Overall, these findings confirm that the impaired transgene induction was predominantly developed, due to the presence of non-

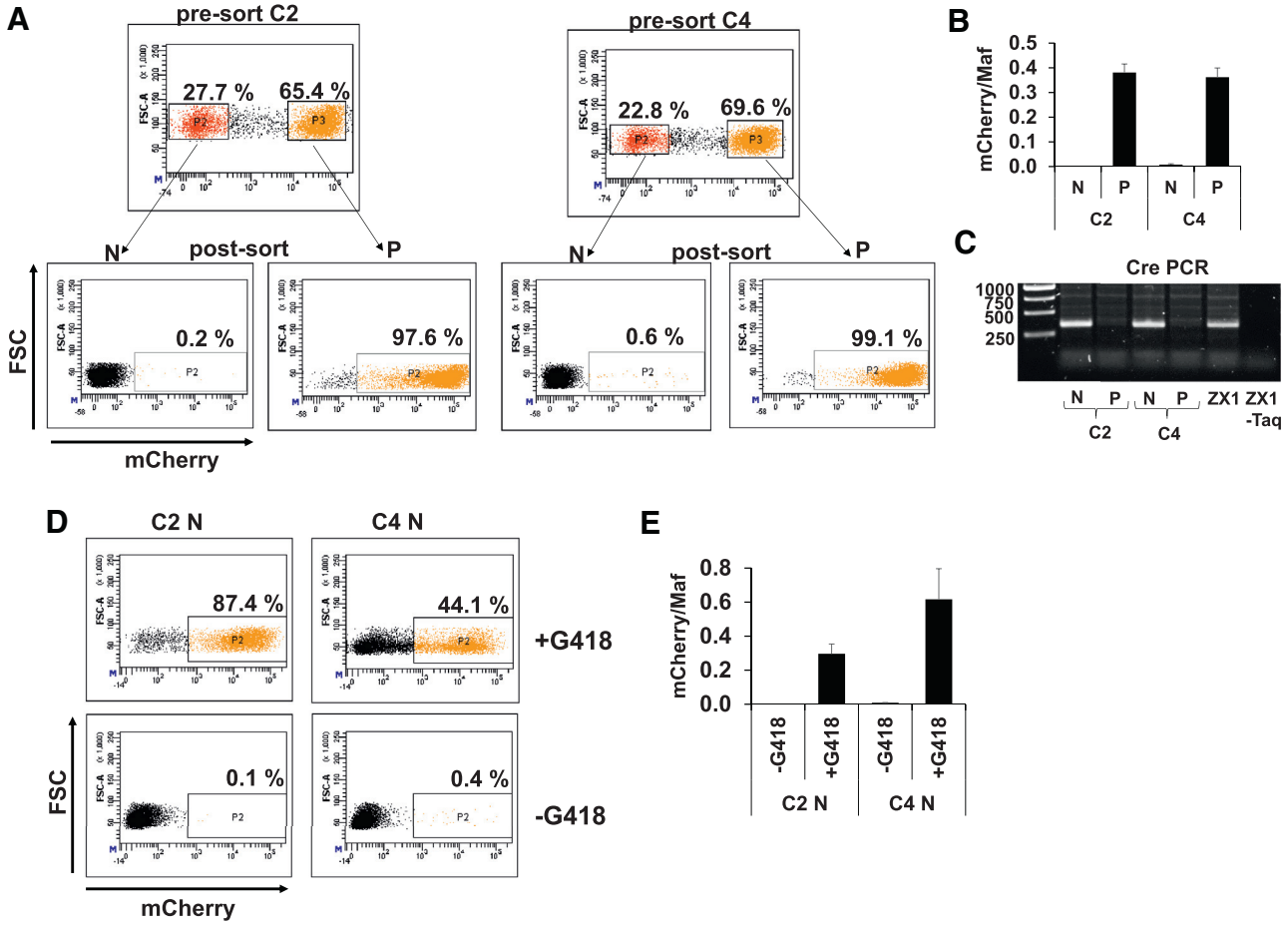

Fig. 4. Genetic heterogeneity of the inducible ES cell clones. (A) Cell sorting of the mCherry carrying ES cell clones (C2 and C4). The mCherry positive 'P' and negative cells 'N' were separated by FACS. Post-sorted cells were further cultured for three days without DOX treatment, thereafter re-stimulated with DOX (1 μg/ml) for additional 2 days. (B) Transgene content of the post-sorted ES cells. Genomic DNA was obtained from the post-sorted reporter positive 'P' and negative cells 'N'. Relative copy number of mCherry was determined by QPCR and normalized to the Maf gene. (C) Detection of the Cre gene with PCR from the sorted cells. Genomic DNA was obtained from the reporter positive 'P' and negative cells 'N'. ZX1 ES cell derived DNA was used as a positive control. (D) Flow cytometric profiles of the post-sorted negative cells 'N' with or without G418 re-selection. The sorted negative 'N' ES cells (C2 and C4) were expanded for 7 days in the presence or absence of 300 μg/ml G418 and re-stimulated with DOX (1 μg/ml) for additional 2 days. (E) Transgene content of the post-sorted and G418 reselected ES cells. Genomic DNA was obtained from the post-sorted reporter negative cells with or without G418 re-selection. Relative copy number of mCherry was determined as described in part B.

recombined cells, however, epigenetic silencing can also attribute to the depressed transgene expression, at least in the G418 re-selected ES cell subsets.

4. Discussion

In this report we have assessed the mCherry induction capacity of a panel of DOX-regulated, ES cell clones at single cell level. This transgene was readily upregulated upon DOX exposure, however, the distribution of the reporter signal was variable among the tested clones. Detecting variegated gene expression, in an apparently homogeneous cell population, is not a novel finding. It was described that higher dose of glucocorticoid led to an increased frequency of cells displaying a high level of reporter expression rather than a uniform increase in expression in each cell (Ko et al., 1990). In addition, heterogeneous gene induction was detected in a DOX-inducible cell culture system (Wong et al., 2005) and variegated EGFP expression was observed in murine ES cells (Ramunas et al., 2007).

Importantly, our inducible ES cell clones firmly maintained their characteristic transgene expression pattern, and the induction capacity of these DOX-responsive cells was propagated to their progenies suggesting that the observed heterogeneous expression is not the consequence of the random fluctuation of gene expression. We found that 10–50% of the ES cells, within the individual clones, failed to express any mCherry specific fluorescence signals. Our analysis revealed that predominantly a genetic alteration was responsible for the impaired transgene induction because the mCherry transgene was missing from the non-responsive cells. There are several possibilities to explain the origin of this mCherry gene free cell population. Our ES cell clones might be contaminated with non-transgenic cells during the cell line establishment or expansion. Our PCR analysis failed to support the ES cell contamination theory because a Cre gene specific PCR product was detected from the non-responsive (negative) cells suggesting that the engineered ES cell clones contained non-recombined ZX1 like cells. These results raise the question how these non-recombined cells survived the antibiotic selection. Of note, we employed a prolonged (7–8 days) G418 treatment during the establishment of the ES cell clones. It is intriguing to speculate that some of the recombined ZX1 cells might only transiently expressed the neomycin resistant gene due to the reversible nature of recombination, and these transiently resistant cells survived the initial selection. Consistent with this possibility, it was described that the neomycin resistance gene can establish resistance to G418 with minimal expression level in mouse ES cells (Nakatake et al., 2013). It is also possible that those ZX1 cells which were located close to a growing resistant ES colony survived the selection because the nearby resistant cells locally decreased the antibiotic concentration. Regardless of the exact mechanism, our data suggested that the initial G418 selection was inefficient to completely eradicate

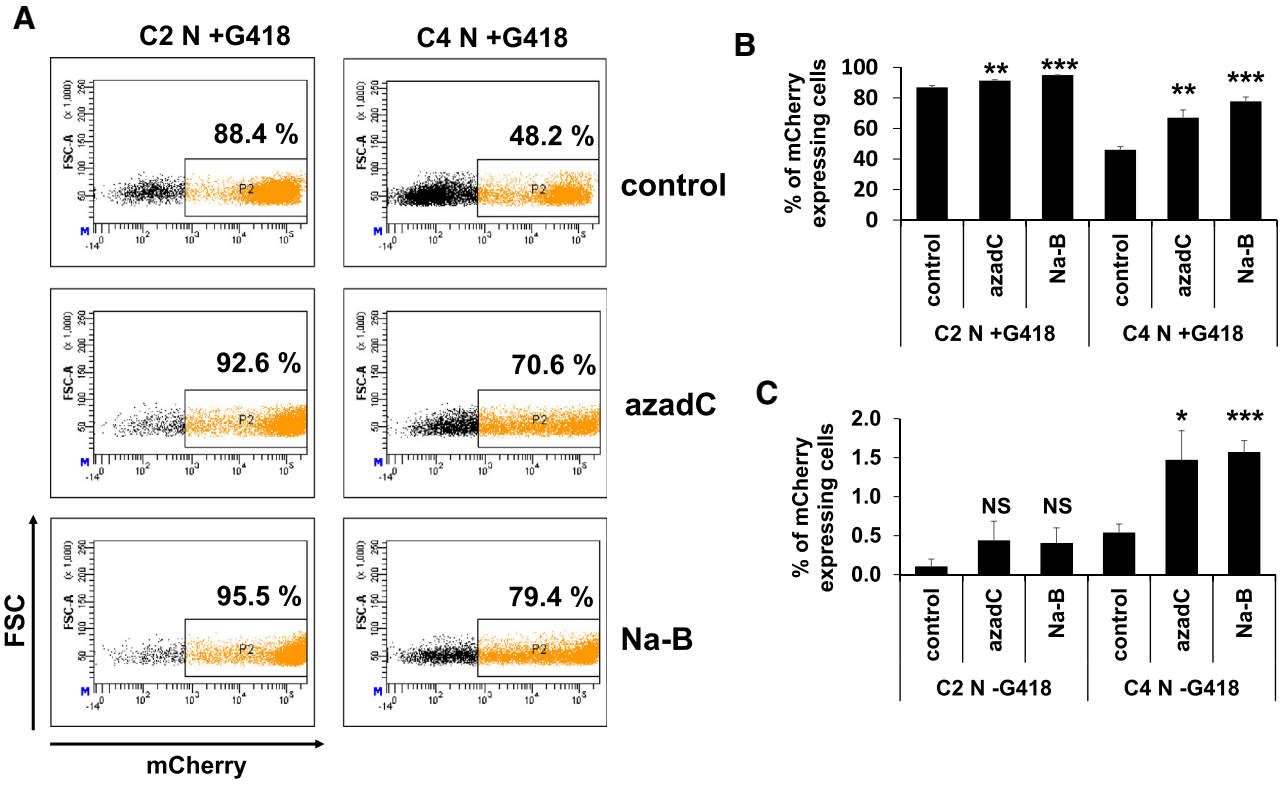

Fig. 5. Elevated transgene expression upon administration of epigenetic modulators. The mCherry specific, sorted negative 'N' ES cell clones (C2 and C4) were expanded for 7 days in the presence or absence of 300 μg/ml G418. Thereafter they were cultured in the absence or presence 50 μM 5-aza-2′-deoxycytidine (azadC) or 20 nM Na-butyrate (Na-B) for 2 days and co-treated with DOX (1 μg/ml). (A) Representative flow cytometric profiles of the negative 'N', but G418 re-selected mCherry specific ES cell clones. (B) Average percentage of the mCherry expressing cells of the G418 re-selected negative clones (N + G418). (C) Average percentage of the mCherry expressing cells of the G418 non-selected negative clones (N − G418). In part B–C the average and the SD values were calculated from 3 independent experiments, in addition, the significant differences between the control and treated (azadC or Na-B) mean values were evaluated using two-tailed, unpaired Student's *t*-test (NS: non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

the non-recombined cells during the establishment of the ES cell clones. To prevent the regeneration of non-resistant cells, permanent chemical selection is frequently applied, using lower concentration of G418 in mammalian transgenic cell lines. However permanent G418 selection cannot be employed automatically in ES cell culture because these pluripotent cells are often maintained on antibiotic sensitive feeder layers. For example, we routinely cultured and expanded our ES cells on G418 sensitive mouse embryonic fibroblast (MEF) feeder cells because we had a limited access to the expensive neomycin resistant MEF. Instead of continuous G418 selection, we recommend a single post-expansion G418 re-selection which can be employed just before ES cell differentiation, using feeder cell free conditions. The other possible way to isolate pure transgenic cell lines is dilution cloning or single cell sorting coupled cloning. However, dilution cloning is rarely used in ES cells; especially it is difficult to apply it in human ES cells, because these cells can poorly survive in single cell stage (Merkert and Martin, 2016).

The G418 selectable genetic heterogeneity, only partially explains the impaired transgene induction capacity of our cells because some of the G418 re-selected cells remained refractory to DOX treatment. We assumed that epigenetic silencing also participated in the repression of the reporter transgene. Consistent with this notion, we found that G418 re-selected ES cells exhibited an increased transgene expression upon DNA demethylation or a histone deacetylase inhibitor (Na-butyrate) treatment suggesting that epigenetic silencing can develop in this genomic region at least in a subset of the cells. Of note, the bacterial backbone of the p2lox targeting vector is integrated into the X chromosome upon recombination using the ICE method (Fig. 1). In addition, it was described that bacterial DNA fragment can initiate epigenetic silencing (Riu et al., 2007). Therefore it is possible that the inserted bacterial DNA fragment can initiate gene silencing despite the fact that the genomic region of Hprt has been considered to be a site at which transgenes express stably (Bronson et al., 1996; Iacovino et al., 2011).

In summary, in this study we observed that a subset of the transgenic ES cells failed to express the reporter genes upon DOX treatment. Importantly, we can eliminate most of these non-responsive cells with G418 re-selection. In addition, it is advocated to sort the DOX-responsive cells using reporter transgenes to further enhance the inducibility of the ES cell clones. Together these improvements allow us to obtain a uniform DOX-inducible cell population at least ex vivo. We suggest that these post-cloning selection strategies can enhance the robustness and the transgene mediated cell conversion capacity of the drug-inducible systems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2016.08.014.

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