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Toxin–antitoxin based transgene expression in mammalian cells

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ABSTRACT

Long-term, recombinant gene expression in mammalian cells depends on the nature of the transgene integration site and its inherent properties to modulate transcription (epigenetic effects). Here we describe a method by which high transgene expression is achieved and stabilized in extensively proliferating cultures. The method is based on strict co-expression of the transgene with an antitoxin in cells that express the respective toxin. Since the strength of antitoxin expression correlates with an advantage for cell growth, the cells with strong antitoxin expression are enriched over time in cultures of heterogeneous cells. This principle was applied to CHO cell lines that conditionally express the toxin kid and that are transduced to co-express the antitoxin kis together with different transgenes of interest. Cultivation of pools of transfectants that express the toxin steadily increase their transgene expression within several weeks to reach a maximum that is up to 120-fold over the initial status. In contrast, average transgene expression drops in the absence of toxin expression. Together, we show that cells conditionally expressing kid can be employed to create overexpressing cells by a simple coupling of kis to the transgene of interest, without further manipulation and in absence of selectable drugs.

INTRODUCTION

To achieve sustained expression of transgenes in mammalian cell lines stable integration into the host genome is the most successful approach. The strength and stability of expression depends strongly on the chromosomal integration site. Integration is mostly random (1). Although methods for targeted integration are emerging (2–5) they have not yet been established as routine procedures in biotechnological applications. Currently, the application of a co-expressed selectable marker and subsequent screening for appropriate expression features is the method of choice (6). Much effort is required to identify the low percentage of cell clones having integrated the transgene in a chromosomal locus that favors expression strength and stability. In industrial set-ups this requirement is met by robots and sophisticated analysis technologies. Accordingly, methods have been developed to facilitate identification of high producer clones (7). The use of drug resistance genes is straight forward. Most selection systems function in a way that the expression strength of the resistance gene, and thus the co-expressed transgene, has to be high enough to detoxify the applied drug concentration. Thus, by theory, high drug concentrations should select for high transgene/selectable gene expression. However, due to side effects of the drug the window for its application is limited. This is why the isolation of highly expressing cell clones purely by selection is not satisfying. Also, the application of selective drugs during the production processes is usually not feasible since it is expensive and needs additional purification and disposal steps.

Bacteria have evolved mechanisms to maintain plasmids with remarkable stability. Toxin/antitoxin systems are known to mediate this stability (8–10). One of the best characterized systems is the *ParD*-system, a toxin/antitoxin-system from *Escherichia coli*, that is constituted by the proteins kis and kid (11). The toxin kid inhibits cell proliferation and the antidote kis antagonizes this inhibition. In bacteria, toxin–antitoxin mechanisms are post-segregational killer systems that eliminate bacteria by selectively killing plasmid-free cells (12). The *ParD* operon is derived from plasmid R1 in Gram-negative bacteria transcribing both the toxin kid (killing determinant) and its antidote kis (killing suppressor). Tightly controlled post-transcriptional processing of the bicistronic mRNAs ensures a balanced expression of the kis over the kid, thereby neutralizing its toxic effect. However, as the antidote kis is comparatively unstable (13), loss of the plasmid during cell division yields in an excess of kid leading to bacterial cell death (14). The parD operon in *E. coli* is tightly controlled by a complex regulatory circuit.

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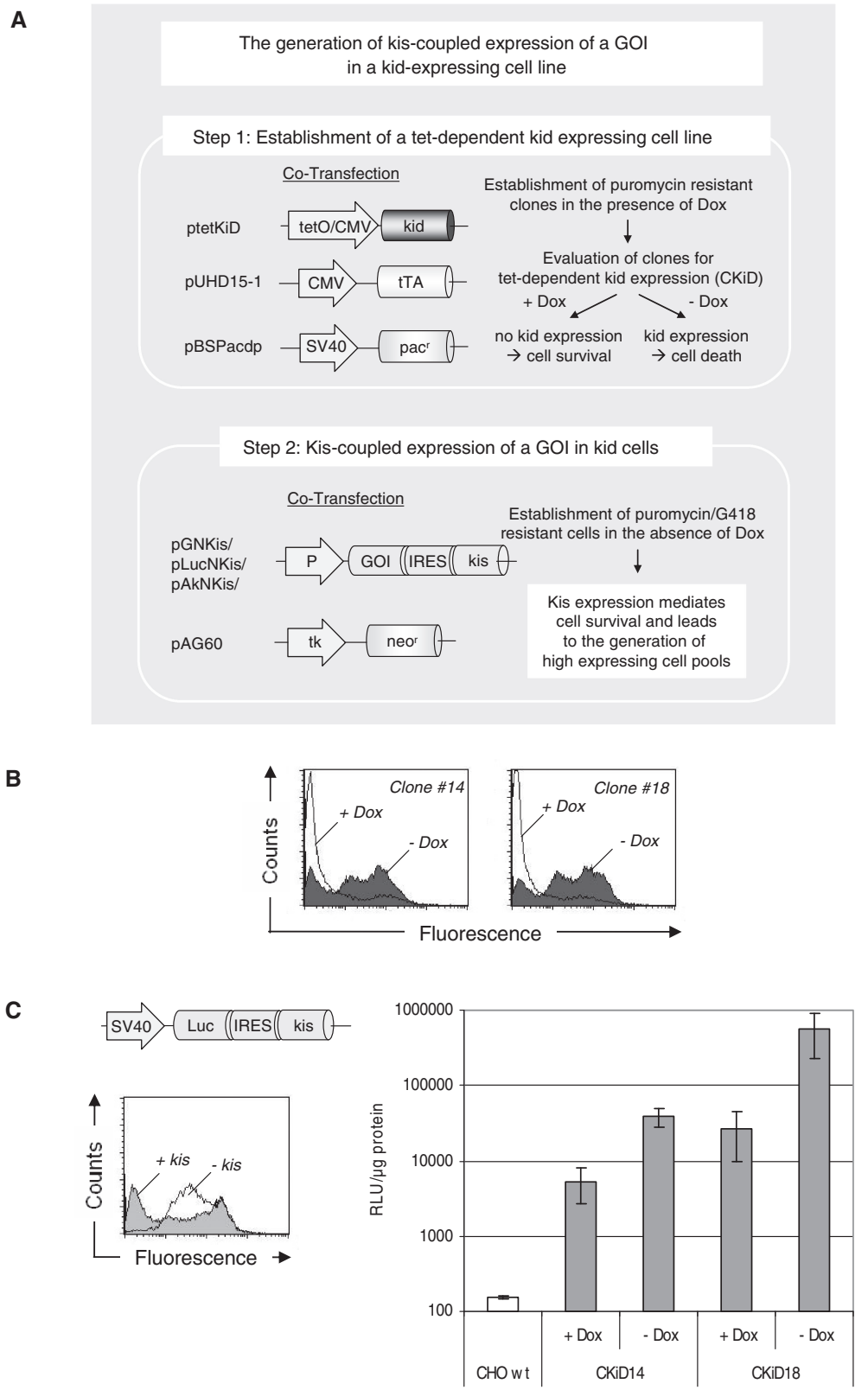

Figure 1. Establishment of the kis/kid system for selection of transgene expression. (**A**) Overall strategy for establishment of regulated kid-expressing CHO-K1 cell clones. To generate conditionally kid expressing cells, cotransfection of a Tet-dependent kid-cassette (ptetKiD), the constitutively expressed transactivator tTA encoded via pUHD15-1 (16) and a puromycin resistance conferring plasmid (pBSBacdp) was applied. To evaluate the kid expression, individual puromycin resistant cell clones were cultivated for 10 days with (+) or without (−) Doxycyclin (Dox). To express the gene of interest (GOI), in a second step, kid expressing cell clones were transfected with an expression vector encoding the GOI (GFP, luciferase and heavy and light chain encoding an antibody, respectively) together with a G418 resistance conferring vector (pAG60). Cells resistant for puromycin

(Continued)

It was shown that the kis/kid killer system can be used to control cell proliferation in different eukaryotic cells from yeast to human (15) because the expression of kid leads to cell growth inhibition and apoptotic cell death.

We applied this principle to the selection of mammalian cells for expression of recombinant genes. The transgene of interest was strictly coupled to the expression of the antitoxin. While pure selection with this system is a long-lasting process a successive enrichment of highly expressing cells was observed. We have refined the system for selection of highly transgene expressing cells and show superior application over existing methods.

MATERIALS AND METHODS

Establishment of kis/kid cell pools and plasmids employed

kis and kid coding sequences were obtained via PCR from plasmid pR1drd19 (Deutsche Sammlung für Mikroorganismen, Braunschweig, DSMZ No. 3880).

ptetKid was generated upon integration of the kid coding sequence into pUHD13-1 (16) thereby controlling its expression by the Tet-dependent transactivation. For the generation of the conditional expressing cell ptetKid was cotransfected together with the tet transactivator coding vector pUHD15-1 ('tet-off') (16) and pBSpacdp (17) which confers puromycin resistance. Cells were selected in presence of puromycin and doxycycline (Dox) (kid-off). Clones 14 and 18 represent puromycin resistant cell clones, sensitive upon withdrawal of Dox for at least 150 days after establishment and initial expansion.

pGNKis, pLucNKis, pAkNKis encode an SV40 promoter driven polycistronic mRNA employing the NRF IRES element. In these mRNAs the reporter genes eGFP, firefly luciferase, or the genes encoding the heavy and light chain of an IgG molecule, respectively, are linked to the kis coding sequence which is placed in the last cistron (18). We note that the expression of the antibody chains is not stoichiometric. Populations of pGNKis, pLucNKis, pAkNKis expressing cells were obtained upon cotransfection with the neomycin phosphotransferase encoding plasmid pAG60 (19) and subsequent selection for G418 resistant cells.

Vector sequences or maps are available upon request.

Mammalian cell culture and transfection

CHO-K1 cells (ATCC CCL 61) were cultivated at 37°C in a humidified atmosphere with 5% CO_2 in Ham's F12 medium (Gibco BRL) with 10% fetal calf serum (Biowest), 2 mM L-glutamine, penicillin (10 U/ml) and streptomycin sulfate (100 μg/ml). Cells were transfected with 1–5 μg of the respective DNA using GenePORTER^{TM}2 Transfection Reagent (Peqlab). Selection was performed in medium supplemented with puromycin (2.5 μg/ml) and/or G418 (500 μg/ml) as specified in Figure 1. Dox was applied in concentrations of 2 μg/ml if indicated.

Evaluation of cell viability and eGFP expression via flow cytometry

FACSCalibur (Becton Dickinson) was used for determination of cell viability in kid expressing cells. The cells were washed, trypsinized and incubated with propidium iodide (50 μg/ml). Dead and proapoptotic cells take up propidium iodide which stains the cellular DNA. Vice versa, live cells cannot take up this dye and remain unstained.

eGFP expression profiles in cell populations was obtained upon propidium iodide staining, thereby excluding dead cells from the analysis.

Luciferase assay

For evaluation of luciferase the cells were seeded in 6-well plates. Cell lysates were created by four freeze-thaw cycles with liquid nitrogen and subsequent centrifugation of cell debris. Ten microliters of the protein lysate (4.8.1) were mixed with 400 μl of reaction buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 1 mM ATP pH 7.8). Fifty microliters of 0.1 mM synthetic D-luciferin (Promega) in 25 mM glycylglycine pH 7.8 was automatically injected in a luminometer [Lumat LB9507 (Berthold)]. Luciferase activity was measured in relative light units (rlu) and corrected for protein content (RLU/μg protein).

ELISA

The specific productivity of the cell populations was analysed by sandwich enzyme linked immunosorbent assay (ELISA). The cells were seeded on a 6-well plate with a density of 5×10^5 cells and incubated for 24 h with 2 ml of medium. The next day the cell number was determined, the supernatant harvested and centrifuged (5 min for 1000 rpm) and added to a 96-well plate covered with an Fc-specific anti-human IgG (SIGMA I2136-1ML). The photometric measurement was done based on a substrate conversion by peroxidase (HRPO) labelled goat anti human IgG (H + L), (CALTAG^{TM} Laboratories, Code No. H10307). An antibody standard was used for calibration. Antibody production was calculated to pg antibody per cell and day (pcd).

Figure 1. Caption continued.
and G418 were generated in the absence of Dox and evaluated for expression of the GOI. (**B**) Two kid expressing cell clones (#14 and #18) are depicted. These cell clones showed normal growth behavior in the absence of kid, cells grown in the presence of kid (−Dox) died or became proapoptotic. Cell death was monitored by propidium iodide-staining and FACS-analysis after 10 days of growth in the absence of Dox. (**C**) *kis mediated rescue:* the indicated bicistronic expression plasmid pLucNKis encoding luciferase and kis was transduced to CKiD clones #14 and #18 cells according to the procedure described in (A). Cell death was monitored after 10 days of cultivation in presence/absence of Dox. Up to 40% of cells of a kid-expressing culture (−Dox) became rescued if compared to non-transfected cells (left). Luciferase activity was measured in kid expressing cells upon cultivation in the presence or absence of Dox for more than 50 days (right). Expression from CHO wild-type cells obtained upon applying the same regimen is depicted for comparison. The data were obtained from three independent measurements.

Real-time quantitative PCR

Total RNA was isolated from a confluent 12-well using RNeasy columns (Qiagen) including a DNase digestion step. For generation of cDNA, 3.3 µg of total RNA was reversely transcribed with 0.5 µg OligodT primer using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). Each cDNA reaction (1.5%) was used as a template for the quantitative analysis of kis mRNA expression (primers 5′-ATACCACCCGACTGAAGA GG-3′ and 5′-CATCCAGCCATTCTCGTTCT-3′). Two-hundred and fifty nanomolars of each primer was used in a standard PCR protocol (15 min 95°C, 45 cycles 15 s 95°C, 20 s 58°C and 30 s 72°C) with the LightCycler® 480 Real-Time PCR System (Roche Applied Science). For each PCR, a no-template reaction was included as negative control. Each cDNA sample was tested in duplicate. Expression levels of kis detected by qPCR were normalized using Eif3i as a reference gene (20). For this purpose the same PCR conditions were employed (primers 5′-CCACAACTTCCACCAGGATT-3′; 5′-ATGCGGAC GTAACCATCTTC-3′). The analysis was done with the LightCycler® 480 Software release 1.5.0 (Roche Applied Science).

RESULTS and DISCUSSION

We developed a mammalian cell expression system that enables the controlled expression of the kid gene in a Tet-off dependent manner. A Tet-regulated kid cassette and a constitutively expressed transactivator (tTA) were stably established in CHO-K1 cells (Figure 1A). Cell clones were isolated and cultivated in the presence of doxycycline (+Dox) to protect the cells from the toxin function. The clones were evaluated for viability and growth in absence of Dox. Toxin function and protection varied within individual cell clones, as some showed a high percentage of dead cells even in the presence of Dox, while others were not sufficiently eradicated in the absence of Dox or lost this property over time. We assume that the strictness of regulation in the various clones strongly depended on the chromosomal integration site of the toxin expression cassette which was reflected by different rates of cell death after Dox removal. Further, a number of cell clones showed retarded growth in the presence of Dox which was interpreted to be indicative for integration sites that do not allow full repression of the toxin. We selected two cell clones that showed nearly unhindered growth in the presence of Dox (see Supplementary Data) and at least 80 % of cell death when cultivated without Dox for 10 days (#14 and 18) (Figure 1B). As exemplified for clone #18, the transfection of a vector coding for the antitoxin kis protects up to 40% of the cells against the kid-mediated cell death (Figure 1C).

We evaluated the potential of this selection system for transgene expression and employed luciferase as a reporter gene. Initial experiments upon cotransfection of two independent vectors encoding luciferase and kis did not show any benefit over conventional methods (data not shown). However, when the firefly luciferase gene was transcriptionally coupled to the kid-antagonist kis by an IRES element, the system showed significant advantages. CHO-K1 cell clones #14 and 18 harboring the well-regulated kid gene were transfected with the bicistronic construct pLucNKis, where luciferase is in the first cistron, followed by the NRF-IRES (18) and the kis gene. Cell pools of stable transfectants were created by co-transfer of a neomycin resistance gene expressing construct. These pools were cultivated in (+) or (−) Dox medium and luciferase expression was determined. In comparison to the luciferase expression of the cell pools grown in the presence of Dox (absence of toxin expression) the expression of luciferase in cells in absence of Dox (presence of the toxin) increased 8- and 28-fold, in the derivatives of clones #14 and #18, respectively (Figure 1C). Moreover, if compared to control cell pools obtained upon standard co-transfection of the same constructs in wild-type CHO cells a 30–3600-fold expression enhancement could be observed.

The increased expression within the pools of kid transfected cells could be due to a selection process for highly expressing clones that are created by random integration of the transgene-kis cassette.To characterize the increase in gene expression in the cell population in more detail, we implemented a corresponding experimental set-up to monitor gene expression on individual cells during the toxin-mediated selection process. The eGFP gene was coupled to kis and pools of stable transfectants were established and cultivated in presence (+) or absence (−) of Dox. CHO control cells established according to the same protocol gave rise to ∼2–5% of GFP expressing cells 20 days after transfection and did not increase upon cultivation for further 60 days (Figure 2A and data not shown). A different picture was obtained upon transfection of kid expressing cells with the same expression vectors: already 20 days after transfection a significantly higher percentage of cells showed GFP expression. Moreover, an increase in expression was observed upon monitoring the expression profile of the cultures over time (Figure 2B). kid expressing cells cultivated with Dox (kid 'off') increased expression of the eGFP gene in a minor population of ∼25% of cells within the entire cell pool for one month and slightly decreased it upon further cultivation. We assume that from the initially established cell pool non-expressing cells are over-growing the producer cells In contrast, cells cultivated without Dox (kid 'on') showed steadily increasing proportions of cells with high levels of GFP in a time-dependent manner for more than 50 days. After this time period only 10% of the non-expressing cell fraction was remaining while the fraction of expressing cells increased accordingly. The mean fluorescence of all cells within the pools of clones #14 and 18 derived cells increased successively to 5- and 16-fold, respectively, if compared to the non-induced pools. This status was not significantly altered during further cultivation of 100 days. It is important to note that high-expressing cells were already found after the initial selection period (7 days), but the toxin-mediated selection increased the percentage of these cells. Thus, the toxin screening seems to select

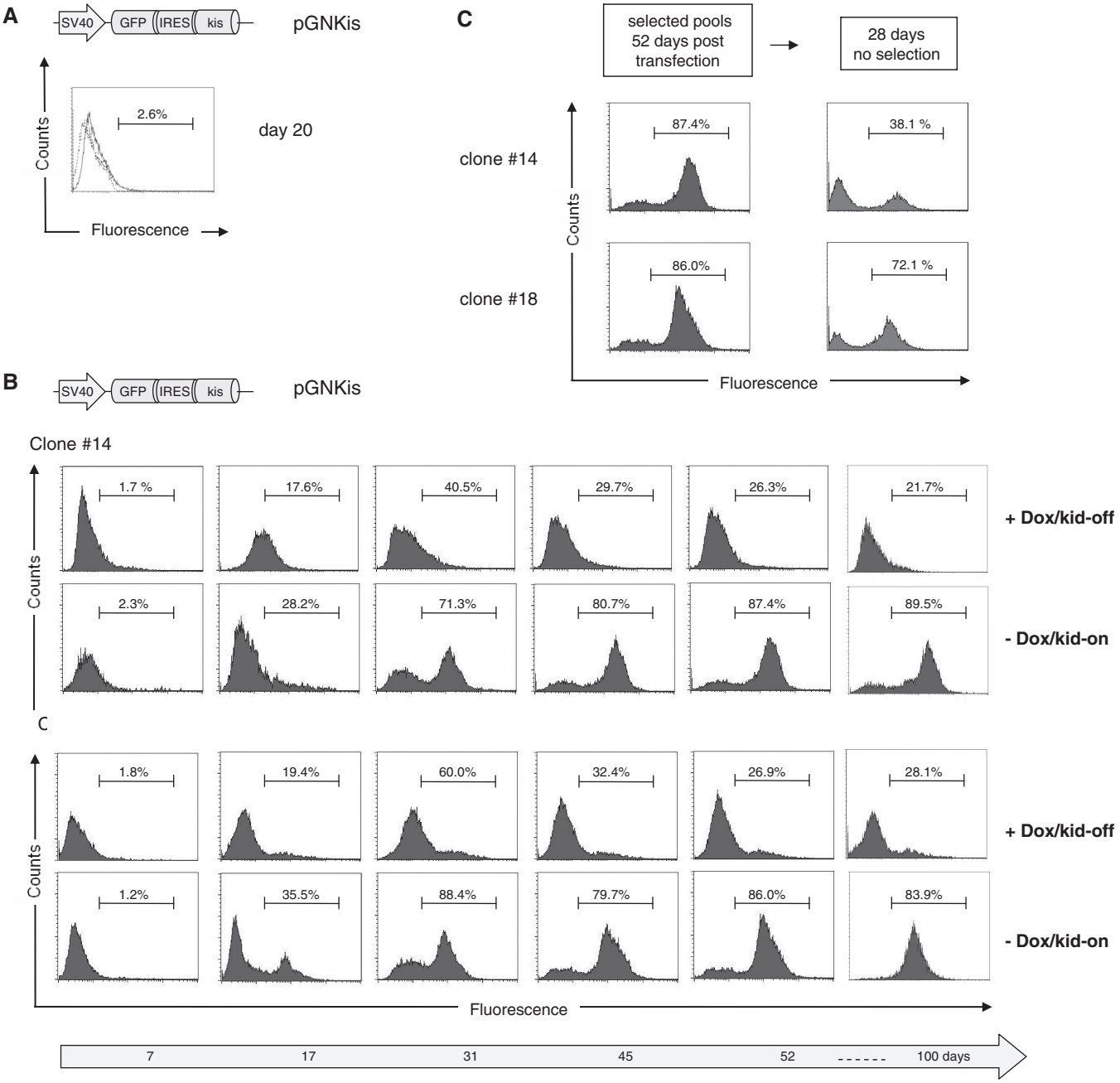

Figure 2. Transgene expression upon coupling to kis. (**A**) eGFP in CHO wild-type cells. CHO wild-type cells were cotransfected with the eGFP expression construct and pAG60. G418 resistant cell pools were generated and evaluated for eGFP expression 20 days after transfection. (**B**) eGFP expression from a kis-coupled expression construct in kid expressing cells The bicistronic eGFP expression construct coupled to kis in pGNKis was cotransfected together with the neomycin-resistance gene into the two kid-regulated cell clones #14 (upper) and #18 (lower). The cells were cultivated with or without Dox for the indicated period in presence of G418 and puromycin. The eGFP expression profile of the cultures was determined by FACS analysis. The numbers above the bars indicate the percentage of eGFP-expressing cells in the respective cell pool. (C) eGFP expression from selected cell pools in absence of selection pressure. Clone #14 and 18 derived eGFP expressing pools generated upon 52 days of cultivation in absence of Dox and presence of G418 and puromycin were subjected to cultivation in media without Dox and any selection drugs. Expression was measured after 28 days of further cultivation.

for highly expressing cells that are created by random integration during gene transfer, while cell clones that show lower expression levels are either eliminated or over-grown.

We evaluated if the high population of expressing cells is dependent on the continuous cultivation in selection media. For this purpose we made use of clones #14 and 18 derived cell pools previously cultivated for 52 days under selection conditions which exhibit >85% of GFP expressing cells. Upon withdrawal of the selection drugs for 28 days, still 38 and 72% of clones #14 and 18 derived cells, respectively, maintained expression of GFP indicating that expression is relatively stable even in the absence of any selective drugs (Figure 2C). We note that

stability of transgene expression in absence of selection drugs varies between individual populations.

As a proof of concept we challenged the kis/kid system with a transgene that opposes mild disadvantages to the expressing cells. For this purpose we used an antibody expression construct that had failed to provide significant production levels in standard selection approaches (data not shown). The above described bicistronic set-up for the antibody gene and the kis selection gene was applied accordingly. The genes encoding the heavy and the light chain of an IgG molecule and the kis gene were arranged in a tricistronic manner to give pAkNKis (Figure 3). This vector was co-transfected together with the neomycin resistance gene to both Tet-dependent kid expressing cell line clone #18 and CHO wild-type cells. Cell pools were generated upon G418 selection. The mean expression rate of secreted antibody from this pool was 0.01 pcd in clone #18. In parallel experiments in CHO-K1 cells, we obtained comparable levels ranging from 0.005 to 0.02 pcd (Figure 3 and data not shown). The clone #18 derived cell pool increased the level of antibody secretion during a cultivation period of 50 days of in the absence of Dox up to 0.99 pcd (Figure 3; see also Supplementary Figure 2). This corresponds to an increase of 100-fold. The improved expression level was further confirmed in a small scale production (see Supplementary Figure 3). The elevated expression level was found to be stable over further 20 days of cultivation (Figure 3). This shows that the refined kis/kid expression system allows not only for the selection of highly expressing cell populations, but also allows stabilization of this population over time. Such kind of effect was never found in conventionally selected cell populations, even if cell clones have been isolated (data not shown).

According to our model, the expression of the expressed genes should parallel the expression of kis. We thus evaluated if the increase in expression of GFP and luciferase was accompanied with a change in the expression level of kis. Indeed, in all pools cultivated in absence of Dox, a significant increase of kis was confirmed (Figure 4).

Molecular analysis of cells after extended selection/cultivation showed that the populations are heterogeneous, however with a differing dominance of specific cell clones (data not shown). We assume that the kis/kid system directs a slow process in which cells that express the antitoxin (and thus the co-expressed transgene) at the highest level succeed. Cells with reduced expression levels are eliminated or are over-grown. This could be explained by adverse effects on cell proliferation by the toxin, even when the antitoxin is sufficiently expressed to allow the growth of cloned cells. Thus, in a heterogeneous population of cells with respect to antitoxin expression the strongest expressing clones will slowly overcome the other cells.

The establishment of conditionally kid-expressing cells for each cell line as exemplified here for CHO cells is a prerequisite for the application of this system. Accordingly, the toxic effect of kid has to be confirmed

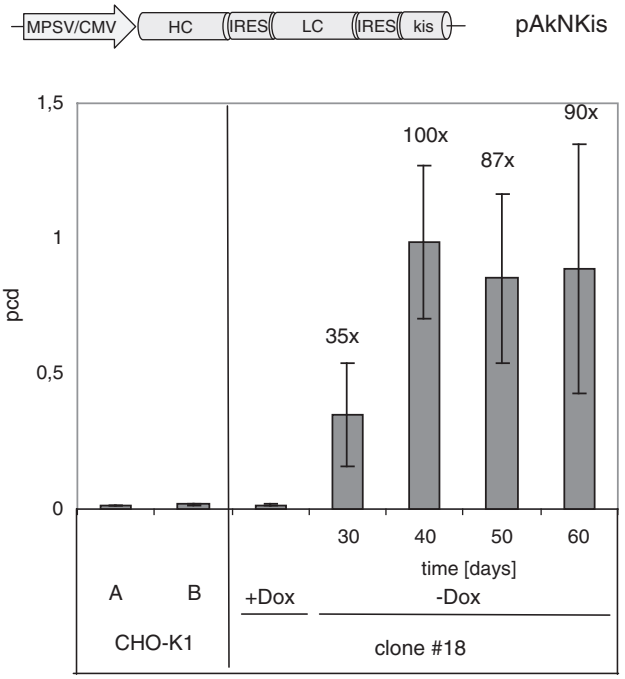

Figure 3. Antibody expression by the kis/kid system: a tricistronic construct pAkNKis from which the heavy and the light chain of an IgG molecule are expressed together with the kis gene was co-transferred together with the neomycin-resistance gene (pAG60) in kid expressing clone #18. As a control, CHO-K1 cells were co-transfected with pAkNKis and pAG60 and selected accordingly. Antibody expression from two independent CHO-K1 populations (A, B) is shown. Clone #18 derived cell pools obtained upon transfection were cultivated for the indicated time in presence of G418 (CHO-K1 transfected cells) or puromycin and G418 for #18 transfected cells, respectively. Antibody expression was monitored after transfection at the indicated time points.The fold increase of expression within clone #18 derived pools cultivated without Dox compared to cells cultivated with Dox is given above the respective bars. Mean values from triplicate samples are depicted.

for the cell line of interest. Further, coupled expression of the gene of interest with kis must be achieved. It is important to note that this strategy can be not only combined with improved expression vectors harboring insulators (21) and S/MARs (22) but also with existing screening procedures [e.g.(7)] including gene amplification (23).

In the presented protocol cotransfection of the kis vector in kid expressing cells was employed. In theory, expression of kis as such should be sufficient to generate stable transfectants even in the absence of any other co-selection gene. When we evaluated this alternative protocol we observed that expression levels of cell pools were lower and less reliable. Thus, for reasons we did not further investigate co-selection revealed to be more robust. Importantly, as we show in Figure 2C, expression is maintained in a high proportion of cells even upon withdrawal of the selection drug and thus does not compromise a drug-free production process.

In summary, expression data from three different proteins (luciferase, eGFP and an antibody) give proof of concept that the toxin/antitoxin strategy can significantly increase the expression level in cell

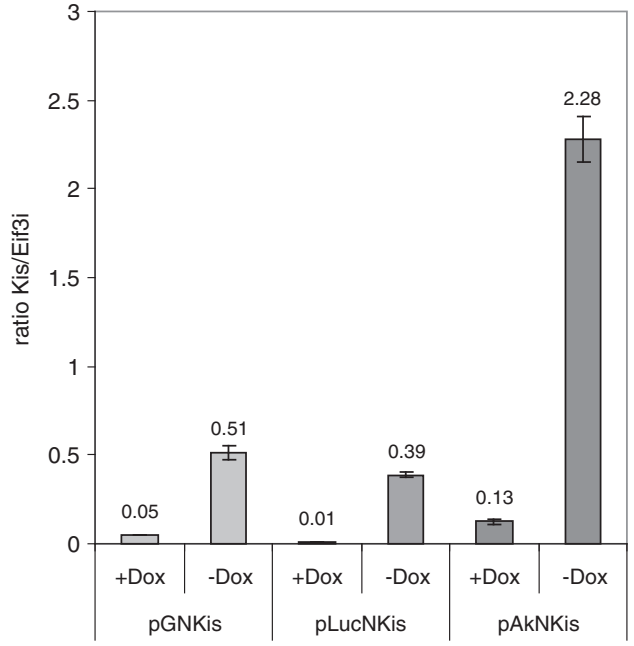

Figure 4. kis mRNA expression. To evaluate kis expression in transfectants, quantitative RT PCR assay for kis was employed. The values show the mean values based on four individual samples. Data were normalized to Eif3i expression as a housekeeping gene.

populations and thereby would be in particular of benefit for hard to express proteins. Although the achieved antibody productivity obtained in the experiments provided here was moderate, we assume that the overall productivity could be significantly enhanced using optimized vector designs and a production optimized CHO cell line. Such a setting should achieve high level protein production.

Besides the exploitation of this principle for protein expression, other applications can be envisaged. One might be found in the area of regenerative medicine were specifically differentiated cells are isolated from *in vitro* expandable stem cells. The conditional expression of kid with a tissue-specifically expressed kis gene should lead to the enrichment and selection of the cell populations of interest and inhibit (oncogenic) growth of cells not expressing the chosen marker(s). The slow action of the kid–mediated selection and its possibility to direct it by adjusting the Dox concentration might be of special interest for this purpose.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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