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Streptogramin- and tetracycline-responsive dual regulated expression of p27^{Kip1} sense and antisense enables positive and negative growth control of Chinese hamster ovary cells

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ABSTRACT

We constructed a dual regulated expression vector cassette (pDuoRex) whereby two heterologous genes can be independently regulated via streptogramin- and tetracycline-responsive promoters. Two different constructs containing growth-promoting and growth-inhibiting genes were stably transfected in recombinant Chinese hamster ovary (CHO) cells that express the streptogramin- and tetracycline-dependent transactivators in a dicistronic configuration. An optimally balanced heterologous growth control scenario was achieved by reciprocal expression of the growth-inhibiting human cyclin-dependent kinase inhibitor p27^{Kip1} in sense (p27^{Kip1}S) and antisense (p27^{Kip1}AS) orientation. Exclusive expression of p27^{Kip1}S resulted in complete G_1-phase-specific growth arrest, while expression of only p27^{Kip1}AS showed significantly increased proliferation compared to control cultures (both antibiotics present), presumably by decreasing host cell p27^{Kip1} expression. In a second system, a derivative of pDuoRex encoding streptogramin-responsive expression of the growth-promoting SV40 small T antigen (sT) and tetracycline-regulated expression of p27^{Kip1} was stably transfected into CHO cells. Expression of sT alone resulted in an increase in cell proliferation, but the expression of p27^{Kip1} failed to provide the expected G_1-specific growth arrest despite having demonstrated expression of the protein. This illustrates the difficulty in balancing the complex pathways underlying cell proliferation control through the expression of two functionally distinct genes involved in those pathways, and how a single-gene sense/antisense approach using pDuoRex can overcome this barrier to complete metabolic engineering control.

INTRODUCTION

Future success of cell and tissue engineering for *ex vivo* expansion will be based on technology managing temporal proliferation control of mammalian cells through well-balanced expression of growth-promoting and growth-suppressing genetic determinants (1). Independent expression control of two different transgenes requires two compatible regulation systems. We have recently adapted the pristinamycin resistance operon of *Streptomyces pristinaespiralis* for use as a mammalian gene regulation system (PipOFF system) (2). The PipOFF system is responsive to a class of clinically licensed antibiotics, the streptogramins (Pyostacin®, Virginiamycin, Synercid®), and has been shown to be compatible with the widely used tetracycline-repressible expression technology (TetOFF system) (2,3). The combination of the PipOFF and TetOFF systems in a two-vector configuration would enable dual regulation required for proliferation management of mammalian cells and other complex molecular interventions.

Within all mammalian cells, regardless of type and origin, positive and negative proliferation control is an exquisitely balanced mechanism which integrates signals from both external (growth factors, mitogens, cell–cell contact) and internal sources (differentiation, DNA repair, apoptosis) and ultimately generates a molecular signal that impinges on the cell cycle (4). The cyclin-dependent kinase inhibitor p27^{Kip1} is crucial for balanced regulation between growth and quiescence in many cell types by binding and inhibiting cyclin D-CDK4/6 and cyclin E/CDK2 complexes responsible for G_1–S transition (5,6). p27^{Kip1} expression is induced in contact-inhibited or mitogen-deprived cells which exit from the cell cycle and enter a quiescent state upon accumulation of this cyclin-dependent kinase inhibitor (CKI) (5,7,8). Tetracycline-responsive overexpression of p27^{Kip1} has been shown to arrest CHO cells in the G_1-phase of the cell cycle (9). Furthermore, downregulation of p27^{Kip1} expression is required for S-phase entry of some cell types, and growth factor-mediated activation of the Ras-Raf1-MEK1-ERK1/2 MAP kinase cascade decreases intracellular p27^{Kip1} levels (10–12). This virtually ubiquitous p27^{Kip1}-based regulation system is even present during the management of host cell proliferation by an infecting virus. For example, the

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small T antigen of SV40 (sT) triggers a decline in $p27^{Kip1}$ levels that enables cell-cycle re-entry of non-dividing target cells (13). Based on these observations in nature, we constructed two different vector-based dual regulation systems: (i) sense and antisense $p27^{Kip1}$, whereby expression of sense $p27^{Kip1}$ shuts down host cell proliferation and expression of antisense $p27^{Kip1}$ alone inhibits endogenous $p27^{Kip1}$ expression, leading to increased cell proliferation; and (ii) $p27^{Kip1}$ and sT, whereby expression of $p27^{Kip1}$ would inhibit host cell proliferation and expression of sT would directly increase proliferation. We tested these two dual regulation systems in CHO cells by stably transfecting the vectors and monitoring growth and cell-cycle profiles of cells following the removal of streptogramin or tetracycline from the cultures. With the former system, it was possible to provide complete proliferation management (growth arrest/growth induction) via mutually exclusive expression of sense or antisense $p27^{Kip1}$ simply by removing streptogramin or tetracycline from the cultures.

MATERIALS AND METHODS

Cell culture, plasmids, transfection, regulating antibiotics, immunofluorescence and SEAP activity assay

Chinese hamster ovary cells (CHO-K1, ATCC: CCL61) and their derivatives were cultivated in FMX-8 medium (Dr F.Messi, Cell Culture Systems, Switzerland) supplemented with 10% fetal calf serum (FCS; PAA Vienna, Austria) unless stated otherwise. The cell line CHO-TWIN1$_{108}$ (CHO-K1 stably transfected with pTWIN1) (2) was cultured in medium that also contained 400 µg/ml of G418 (Gibco BRL, Life Technologies). For stable transfection of CHO-TWIN1$_{108}$ with pDuoRex plasmids, the desired dual regulation plasmids were cotransfected at a ratio of 1:15 with pZeoSV2 (Invitrogen) which confers resistance to 100 µg/ml zeocin. For both transient and stable transfections, 6 µg of DNA was used to transfect 400 000 cells at a transfection efficiency of 35 ± 5% (determined by parallel β-Gal-based assays) using an optimized $CaPO_4$ protocol (9). Mixed stable populations were grown in the presence of pristinamycin (2 µg/ml) and tetracycline (2 µg/ml) to repress the dual regulated expression unit and subsequently cloned using FACS-mediated single cell sorting (FACStar^{Plus}; Beckton Dickinson). Growth characteristics of CHO-233 and CHO-226 cell lines were assessed by seeding 100 000 cells in T25 flasks containing FMX-8 medium supplemented with 10% FCS and the appropriate regulating antibiotics (2 µg/ml) (Figs 2 and 4). The cell number was determined daily as an average taken from three independent cultures using a Casy1® cell counter according to the manufacturer's protocol (Schärfe System, Germany).

Plasmid pW2(t^+T^+) encoding the SV40 small and large T antigens was kindly provided by K.Rundell (13). pMF99 encodes the human $p27^{Kip1}$ cDNA (14). Vector pTWIN1, encoding the pristinamycin (PIT)- and tetracycline (tTA)-dependent transactivators (P_{SV40}-PIT-*IRES*-tTA-pA), the dual regulation vector pDuoRex1 ($P_{hCMV*-1}$-MCSI-*IRES*-MCSII-pA_I-/-P_{PIR}-MCSIII-pA_{II}), the multicistronics expression vector pTRIDENT1 and pTBC-1 have been described previously (2,9). pECFP-C1 and pEYFP-C1 were purchased from Clontech, and pcDNA3.1/V5/His-TOPO was purchased from Invitrogen.

Tetracycline (Sigma) and pristinamycin (Pyostacin®; Aventis Inc.) were used as regulating antibiotics at concentrations of 2 µg/ml. Immunofluorescence was performed as described previously (9) using antibodies specific for $p27^{Kip1}$ (M-197 or F-8; Santa Cruz) and sT (Pab108; Santa Cruz) along with FITC- or Texas Red-labeled secondary antibodies (Jackson Immuno Research). Immunofluorescence was visualized using a Leica DM-RB fluorescence microscope equipped with appropriate filters. The fluorescence proteins enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) were visualized with the same microscope but using different filters XF104 (ECFP) and XF114 (EYFP) (Omega Optical Inc.).

FACS analysis

For FACS-mediated cell-cycle analysis, cells were washed twice with PBS prior to detachment using dissociation solution (Sigma) and centrifugation at 1500 *g*. The supernatant was removed and the cells were resuspended in PBSAz (0.1% NaAz and 2% FCS in PBS) and centrifuged again. After a second washing step, 10^6 cells were dissolved in 750 µl of ice-cold PBSAz. While vortexing, 250 µl of 1% PFA in PBS was added and the mixture was incubated at 4°C for 1 h. Following centrifugation the cells were dissolved in 1 ml of 0.2% Tween-20 in PBS and incubated for 15 min at 37°C. Cells were then washed three times with PBSAz before incubation in 500 µl propidium iodide staining medium (10 mg/l propidium iodide and 1 mg/l RNAse A in PBSAz) for 30 min at 37°C in the dark. Analysis was performed on an EPICS ELITE apparatus (Beckmann-Coulter) using the MultiCycle software (Phoenix Flow Systems, Inc.).

Western blot analysis

For preparation of whole cell extracts, cells were washed with ice-cold PBS and scraped in ice-cold NP-40 extraction buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 1% NP-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate). Suspensions were incubated 5 min on ice and the lysates were centrifuged at 23 000 *g* for 15 min at 4°C. Protein concentrations were determined using a Bradford assay (Bio-Rad). Whole cell extracts were denatured by boiling in sample buffer and subsequent SDS–polyacrylamide gel electrophoresis (15% PAGE). The proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking with 5% non-fat dry milk (Bio-Rad) in TTBS (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20), filters were probed with specific antibodies ($p27^{Kip1}$, F-8; sT, Pab108). Proteins were visualized with HRP-conjugated donkey anti-mouse secondary antibodies using the ECL detection system (Amersham).

Vector construction

pDuoRex3 (pMF196) contains converging pristinamycin- and tetracycline-responsive expression units and was constructed following a multiple step cloning procedure: (i) the gene encoding ECFP was amplified from pECFP-C1 (Clontech) using oligonucleotides OMF96: GATC**GAATTC**cctcagcaccaggtCATGCTTAAGTCGCGACATATGgatccgctagcgctaccg and OMF89: GATC**AAGCTT/GCCCGGGC**CACACAAAAAACCAACACACAGATGTAATGAAAATAAAGATATTTTATTTGATCAGGCGCGCC**GCGGCCGC**ATGCttacttgtacagctcgtc, and cloned into pcDNA3.1/V5/His-TOPO

(Invitrogen) under the control of P_{CMV} to yield plasmid pSAM228. (ii) The gene encoding the EYFP was amplified from pEYFP-C1 (Clontech) with oligos OMF90: GTAC**GAATTC**GATATCATGCATGGCGCCGTTTAAACGCGTATTTAAATgatccgctagcgctaccg and OMF91: GATCAAGCTTGCGGCCGCGGATCC**GCCCGGGC**CACACAAAAAACCAACACACAGATGTAATGAAAATAAAGATATTTTATTATCGATACTAGTGCGATCGCTTAATTAATTTAAATttacttgtacagctcgtcc, and cloned into pcDNA3.1/V5/His-TOPO under the control of P_{CMV} to result in plasmid pSAM222. OMF89, OMF90, OMF91 and OMF96 contain a variety of restriction sites which flank ECFP and EYFP and enable straightforward replacement of these fluorescence determinants by other genes of interest. OMF89 and OMF90 contain artificial polyadenylation sites (*pA**) in their 3′ extensions (underlined). (iii) The EYFP-*pA** containing cassette was excised from pSAM222 by *Eco*RI/*Not*I (contained in the oligos; bold) and ligated to the corresponding sites of pMF164 (*Not*I/*Eco*RI) thereby replacing GFP of pMF164 and resulting in plasmid pSAM226. (iv) The P_{PIR}-EYFP-*pA** cassette was excised with *Ssp*I/*Not*I from pSAM226 and ligated into the corresponding sites of pTRIDENT1 (pMF125) (9), hence replacing $P_{hCMV*-1}$ and *IRES* I of pTRIDENT1 to give plasmid pMF190. (v) The ECFP-*pA** cassette was excised from pSAM228 by *Eco*RI and *Hin*dIII (contained in the oligos; bold) ligated into the corresponding sites (*Eco*RI/*Hin*dIII) of pTBC-1, resulting in plasmid pSAM227. (vi) The ECFP-*pA** containing cassette was released from pSAM227 by digestion with *Xho*I (contained 5′ of $P_{hCMV*-1}$ in pTBC-1) and *Srf*I (contained in OMF89; bold), and was subsequently ligated to pMF190 restricted with *Sal*I (compatible to *Xho*I) and *Srf*I to result in plasmid pDuoRex3 (pMF196: P_{PIR}-YFP-pA_I-/-pA_{II}-CFP-$P_{hCMV*-1}$).

For the construction of $p27^{Kip1}$ sense–antisense expression vector pMF226, the ECFP-EYFP cassette was excised from pMF196 (pDuoRex3) by *Eco*RI/*Hin*dIII and replaced by the *Eco*RI/*Hin*dIII fragment of pMF99 (14) which encodes the cyclin-dependent kinase inhibitor. pMF226 contains a P_{PIR}→$p27^{Kip1}$←$P_{hCMV*-1}$ sense–antisense expression unit.

pMF233 was constructed by a two-step cloning procedure: (i) sT was amplified from pW2(t^+T^+) (13) using oligos OMF104: GATCGATATCACTAGTgcctaggcttttgcaaaaagc and OMF106: GATCAGATCTACGCGTttagagctttaaatctctg and ligated in sense orientation into pcDNA3.1/V5/His-TOPO (Invitrogen) to result in pMF231. (ii) sT was excised from pMF231 with *Spe*I/*Bgl*II and ligated into the corresponding sites of pMF194 (*Spe*I/*Bgl*II) resulting in pMF233 ($P_{hCMV*-1}$-$p27^{Kip1}$-pA_I-/-P_{PIR}-sT-pA_{II}).

RESULTS

Construction of a dual regulated expression unit

Previous results have shown that the streptogramin- and tetracycline-responsive gene expression systems are compatible and can be used for independent control of two different gene activities (2). Gene therapy and tissue engineering applications require a compact design of expression vectors. The antibiotic-responsive dual regulated expression system consists of PIT and tTA and their responsive promoters P_{PIR} (streptogramin-responsive promoter) and $P_{hCMV*-1}$ (tetracycline-responsive promoter) (2,3). PIT and tTA were previously cloned in a dicistronic constitutive expression configuration (pTWIN1; P_{SV40}-PIT-*IRES*-tTA; IRES, picornaviral internal ribosomal entry site) then stably expressed in a CHO cell line, resulting in CHO-TWIN1$_{108}$ (2). The cloning of the streptogramin- and tetracycline-responsive expression units was more challenging because careful consideration of orientation of the two genes with respect to each other was required. Three orientations were examined: (i) divergent (←/→), (ii) convergent (→/←) and (iii) consecutive (→/→). Divergent configurations showed undesired co-regulation of P_{PIR}- and $P_{hCMV*-1}$-driven expression units (as exemplified by bidirectional tetracycline-responsive promoters; data not shown) (15). When P_{PIR}-YFP (yellow fluorescent protein) and $P_{hCMV*-1}$-CFP (cyan fluorescent protein) were oriented in a convergent orientation (pDuoRex3, P_{PIR}-YFP-pA_I-/-pA_{II}-CFP-$P_{hCMV*-1}$) only YFP_{OFF}/CFP_{OFF}, YFP_{ON}/CFP_{OFF} and YFP_{OFF}/CFP_{ON} expression scenarios were achieved (Fig. 1). The YFP_{ON}/CFP_{ON} expression configuration was not possible perhaps due to mutual transcriptional

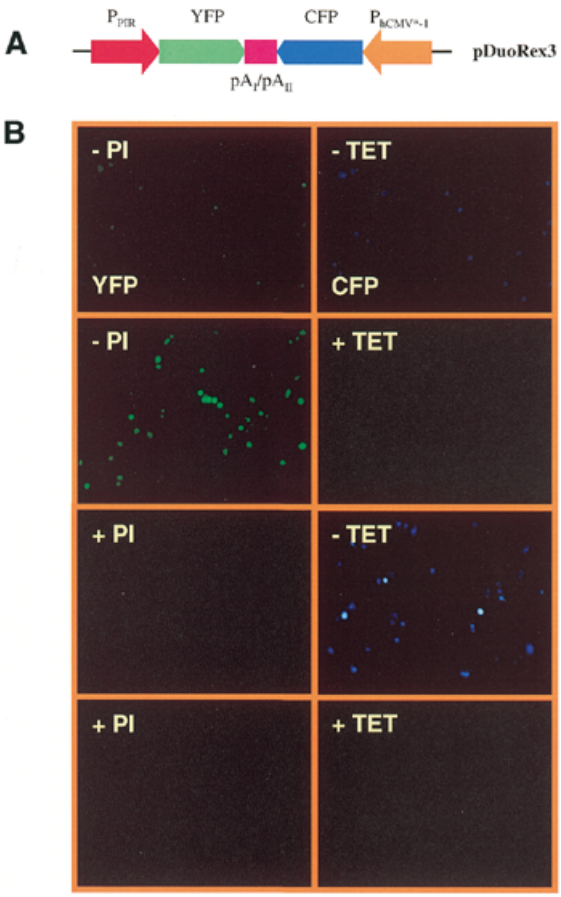

Figure 1. (**A**) Diagram of the dual regulated expression vector pDuoRex3 containing convergently oriented P_{PIR}-YFP-pA_I and $P_{hCMV*-1}$-CFP-pA_{II} expression units (P_{PIR}-YFP-pA_I-/-pA_{II}-CFP-$P_{hCMV*-1}$). (**B**) pDuoRex3 was transfected into CHO-TWIN1$_{108}$, which stably harbors both tTA and PIT-dependent transactivators, and expression of YFP and CFP was assessed 48 h following transfection by fluorescence microscopy in the presence (+) or absence (–) of either tetracycline (TET; 2 µg/ml) or pristinamycin I (PI; 2 µg/ml). In the absence of both antibiotics (–TET/–PI) simultaneous induction of both responsive promoters results in reciprocal transcriptional silencing of the convergently oriented expression units.

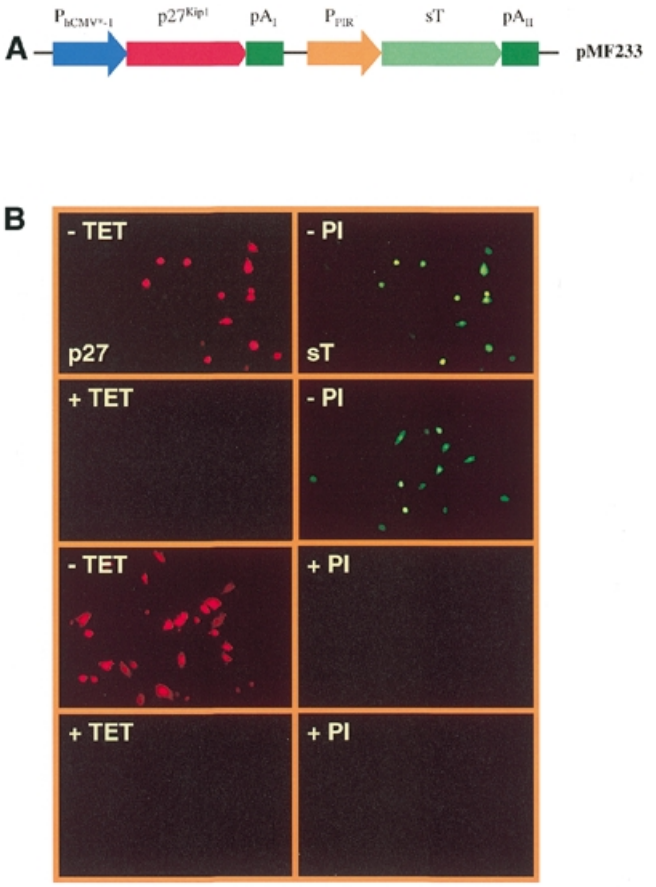

Figure 2. (**A**) Diagram of the dual regulated expression vector pMF233 encoding the human $p27^{Kip1}$ cDNA under the control of the tetracycline-dependent promoter ($P_{hCMV*-1}$) and sT under the control of the pristinamycin-responsive promoter (P_{PIR}). (**B**) Immunofluorescence specific for $p27^{Kip1}$ and sT proteins shows independent regulation of $p27^{Kip1}$ and sT expression in CHO-TWIN1$_{108}$ (constitutively expressing the pristinamycin- and tetracycline-dependent transactivators) cells transiently transfected with pMF233 in response to addition or withdrawal of tetracycline (+/– TET) or pristinamycin (+/– PI).

silencing (Fig. 1). Only pDuoRex1 derivatives harboring both expression units in consecutive orientation ($P_{hCMV*-1}$-$p27^{Kip1}$-pA_I-/-P_{PIR}-sT-pA_{II}) enabled all four regulation scenarios (Fig. 2; see below).

Opposite control of $p27^{Kip1}$ and sT in CHO cells

The human cyclin-dependent kinase inhibitor $p27^{Kip1}$ and sT are theoretically an ideal combination for opposing proliferation control since overexpression of $p27^{Kip1}$ has been shown to arrest CHO cells in the G_1-phase of the cell cycle (9), and sT exerts positive proliferation control by decreasing $p27^{Kip1}$ levels (13). We constructed a pDuoRex1 derivative pMF233 which contains a $P_{hCMV*-1}$-driven $p27^{Kip1}$ expression unit and a P_{PIR}-driven sT in consecutive orientation (Fig. 2A; $P_{hCMV*-1}$-$p27^{Kip1}$-pA_I-/-P_{PIR}-sT-pA_{II}). Transient transfection of pMF233 into CHO-TWIN1$_{108}$ [stably expressing the P_{SV40}-PIT-*IRES*-tTA expression unit (2)] confirmed independent expression of $p27^{Kip1}$ and sT in all four expression configurations: $p27^{Kip1}{}_{ON}$/sT_{ON} (–Tet/–PI); $p27^{Kip1}{}_{OFF}$/sT_{ON} (+Tet/–PI); $p27^{Kip1}{}_{ON}$/sT_{OFF} (–Tet/+PI); $p27^{Kip1}{}_{OFF}$/sT_{OFF} (+Tet/+PI) by immunofluorescence without any signs of crossregulation (Fig. 2B).

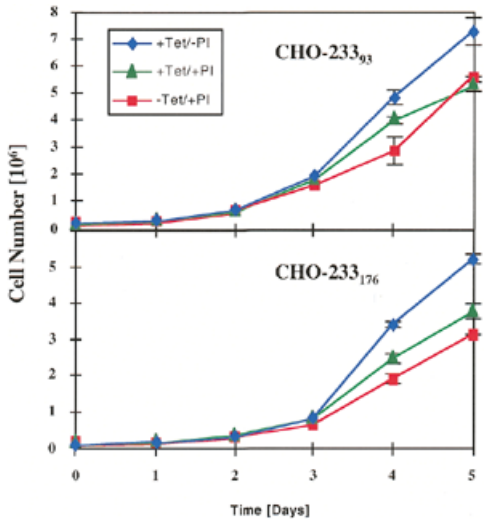

Figure 3. Growth profile of CHO-233 cells expressing $p27^{Kip1}$ or sT. Cell lines CHO-233_{93} and CHO-233_{176} harboring the dual regulation plasmid pMF233 were seeded in triplicate cultures at 10^5 cells/ml under three different conditions: $p27^{Kip1}{}_{OFF}$/sT_{OFF} (+Tet+PI), $p27^{Kip1}{}_{OFF}$/sT_{ON} (+Tet/–PI) and $p27^{Kip1}{}_{ON}$/sT_{OFF} (–Tet/+PI), where +Tet and +PI are cultures containing tetracycline and pristinamycin, respectively (Fig. 1).

For construction of stable pMF233-containing CHO-TWIN1$_{108}$ cell lines both expression units of pMF233 were repressed by addition of pristinamycin (2 µg/ml) and tetracycline (2 µg/ml) during transfection and clonal selection. CHO-233_{93} and CHO-233_{176}, two randomly chosen clones out of 10 $p27^{Kip1}$- and sT-expressing clones were used for further analysis. In order to evaluate the potential of pMF233 to provide complete proliferation control of CHO cells, the growth characteristics and cell-cycle profiles of CHO-233_{93} and CHO-233_{176} were analyzed under mutually exclusive $p27^{Kip1}$ and sT expression scenarios [$p27^{Kip1}{}_{OFF}$/sT_{ON} (+Tet/–PI); $p27^{Kip1}{}_{ON}$/sT_{OFF} (–Tet/+PI)] (Figs 3 and 4). Although expression of sT alone ($p27^{Kip1}{}_{OFF}$/sT_{ON}) increased proliferation of both CHO-233 cell clones, expression of only $p27^{Kip1}$ ($p27^{Kip1}{}_{ON}$/ sT_{OFF}) did not result in desired growth arrest of CHO-233_{93} or CHO-233_{176} clones (Fig. 3). Cell-cycle analysis of CHO-233_{176} confirmed the absence of a G_1-specific growth arrest in the $p27^{Kip1}{}_{ON}$/sT_{OFF} (–Tet/+PI) configuration compared to the $p27^{Kip1}{}_{OFF}$/ sT_{OFF} (+Tet/+PI) control set-up and the isogenic parental cell line CHO-TWIN1$_{108}$ (Fig. 4). As expected, increased proliferation observed under $p27^{Kip1}{}_{OFF}$/sT_{ON} conditions did not alter the cell-cycle distribution relative to the CHO-TWIN1$_{108}$ control (Fig. 4). Since enforced expression of human $p27^{Kip1}$ has previously been shown to arrest CHO cells in the G_1-phase of the cell cycle (9), it is possible that in this $p27^{Kip1}{}_{ON}$/sT_{OFF} scenario, induction of $p27^{Kip1}$ results in leaky expression of the downstream sT expression and/or P_{PIR} shows some basal activity. In both cases, the strong oncogene sT may override $p27^{Kip1}$-mediated arrest signals or prevent $p27^{Kip1}$ expression to reach arrest-specific concentrations (13). Indeed, western blot analysis shows that significant leaky expression of sT correlates with induction of $p27^{Kip1}$ expression (Fig. 5). It is thus clear that the balance between the basal expression of the growth-promoting determinant and $p27^{Kip1}$ expression are crucial for successful proliferation

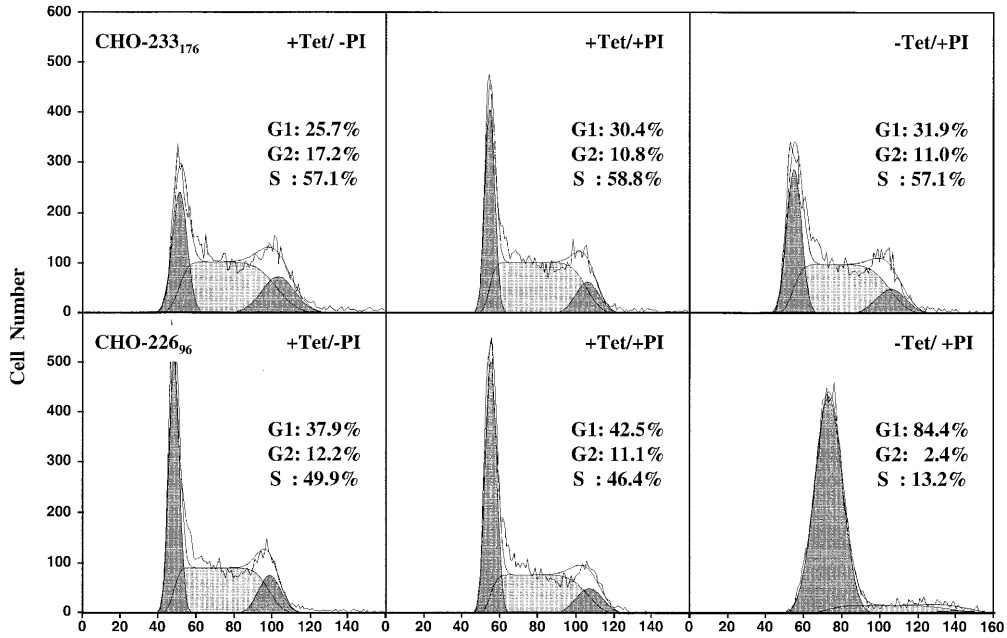

Figure 4. FACS-mediated cell-cycle analysis of CHO-233_{176} and CHO-226_{96}. Both cell lines were grown for 48 h under conditions allowing for all three expression configurations +Tet/–PI (growth-promoting gene induced, sT for CHO-233_{176} and $p27^{Kip1}$AS for CHO-226_{96}), +Tet/+PI (both transgenes repressed) and –Tet/+PI [growth-arresting gene ($p27^{Kip1}$) induced] and incubated with the DNA-staining chemical propidium iodide for DNA content-based assessment of cell-cycle distribution. The cell-cycle profiles of proliferation-enhanced (+Tet/–PI) conditions and set-ups which repress the dual regulation unit (+Tet/+PI) are compared to the cell-cycle distribution of the parental cell line CHO-$TWIN1_{108}$ (G_1, 29.5%; G_2, 10.3%; S, 60.2%). In contrast to CHO-226_{96} cells which arrest in the G_1-phase under –Tet/+PI conditions, CHO-233_{176} cells do not display any accumulation of this cell-cycle phase (see also Figs 3 and 5).

control independent of the combination of growth-promoting and growth-inhibiting determinants used. To overcome these complex issues, a single gene-based strategy for complete proliferation management of CHO cells was designed around $p27^{Kip1}$.

Complete proliferation control of CHO cells by $p27^{Kip1}$-sense and $p27^{Kip1}$-antisense expression

Based on the convergent dual regulated expression vector pDuoRex3 (Fig. 1) we constructed pMF226 which encodes human $p27^{Kip1}$ driven in sense ($p27^{Kip1}$S) orientation by $P_{hCMV*-1}$ and in antisense ($p27^{Kip1}$AS) orientation by P_{PIR} ($P_{hCMV*-1}$→$p27^{Kip1}$←P_{PIR}; Fig. 6A). Success of the convergent dual regulated expression configurations would be most desirable because they are inherently less sensitive to the leakiness of antibiotic-responsive promoters than consecutive set-ups because the expressed unit transcriptionally silences its repressed counterpart.

During the transfection and selection procedure, the dual expression units were repressed by addition of pristinamycin (2 µg/ml) and tetracycline (2 µg/ml). CHO-226_{96}, CHO-226_{100} and CHO-226_{108}, three randomly chosen clones out of 20 $p27^{Kip1}$-expressing clones, were used for further studies. The $p27^{Kip1}$ expression profiles $p27^{Kip1}S_{ON}$/$p27^{Kip1}AS_{OFF}$ (–Tet/+PI), $p27^{Kip1}S_{OFF}$/$p27^{Kip1}AS_{OFF}$ (+Tet/+PI) and $p27^{Kip1}S_{OFF}$/$p27^{Kip1}AS_{ON}$ (+Tet/–PI) were analyzed by $p27^{Kip1}$-based immunofluorescence, a quantitative measure of $p27^{Kip1}$ accumulation 48 h after induction (shown for CHO-226_{96} in Fig. 6). Significant overexpression of $p27^{Kip1}$ is observed upon sense expression and intracellular levels of this CKI are reduced below endogenous levels upon exclusive antisense expression of $p27^{Kip1}$ (Fig. 6B). Analysis of the growth profiles of CHO-226_{96}, CHO-226_{100} and CHO-226_{108} demonstrated that $p27^{Kip1}$S expression successfully arrests proliferation of these engineered CHO derivatives for extended periods (Fig. 7). However, upon exclusive expression of antisense $p27^{Kip1}$, all three cell clones displayed significantly increased proliferation, reaching almost twice the cell density of CHO-226 clones grown in the presence of pristinamycin and tetracycline (control culture, $p27^{Kip1}S_{OFF}$/$p27^{Kip1}AS_{OFF}$) (Fig. 7). FACS-mediated cell-cycle analysis of CHO-226_{96} clearly confirmed a G_1-specific growth arrest under $p27^{Kip1}S_{ON}$/$p27^{Kip1}AS_{OFF}$ conditions while increased proliferation observed with $p27^{Kip1}S_{OFF}$/$p27^{Kip1}AS_{ON}$ configurations did not alter the overall cell-cycle distribution compared to control set-ups (CHO-$TWIN1_{108}$ and $p27^{Kip1}S_{OFF}$/$p27^{Kip1}AS_{OFF}$) (Fig. 4).

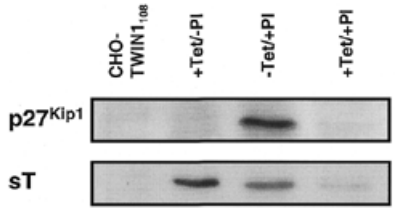

Figure 5. Western blot analysis of CHO-233_{176}. $p27^{Kip1}$ and sT expression of CHO-233_{176} was analyzed in the presence (+) and absence (–) of tetracycline (Tet) or pristinamycin I (PI). Owing to the consecutive orientation, induction of $p27^{Kip1}$ significantly increases leaky expression of sT.

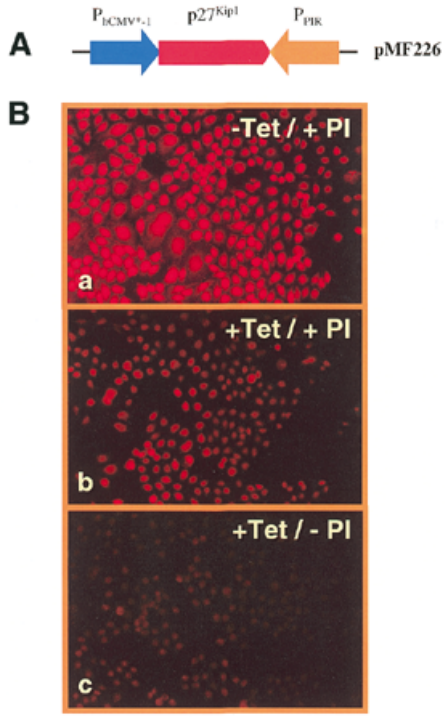

Figure 6. (**A**) Schematic of the p27^{Kip1} sense/antisense expression vector pMF226. p27^{Kip1} sense expression is driven by the tetracycline-responsive promoter ($P_{hCMV*-1}$) and p27^{Kip1} antisense expression is controlled by the PI-responsive promoter (P_{PIR}). (**B**) p27^{Kip1}-based immunofluorescence of the pMF226-harboring cell line CHO-226$_{96}$ expressing (a) sense p27^{Kip1} only (–Tet/+PI), (b) neither gene (+Tet/+PI, complete repression) and (c) antisense p27^{Kip1} only (+Tet/—PI). The images represent a quantitative measure of p27^{Kip1} accumulation after 48 h of induction.

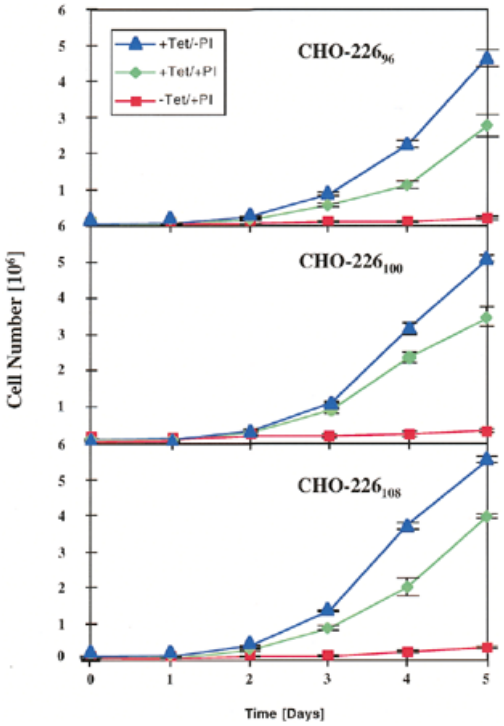

Figure 7. Growth profiles of CHO-226 cells expressing p27^{Kip1}S or antisense p27^{Kip1}AS. Three clones (CHO-226$_{96}$, CHO-226$_{100}$, CHO-226$_{108}$) harboring the plasmid pMF226 ($P_{hCMV*-1}$-p27^{Kip1}-P_{PIR}) were seeded in triplicate cultures at an initial population of 10^5 cells/ml under three different conditions: p27$^{Kip1}S_{OFF}$/p27$^{Kip1}AS_{ON}$ (+Tet/–PI), p27$^{Kip1}S_{ON}$/p27$^{Kip1}AS_{OFF}$ (–Tet/+PI), or p27$^{Kip1}S_{OFF}$/p27$^{Kip1}AS_{OFF}$ (+Tet/+PI, complete repression of both genes).

DISCUSSION

Advances in biomedicine have accentuated the use of gene therapy as an attractive platform for the delivery of a therapeutic protein (16,17). Human-compatible gene regulation technology will become an increasingly important option for pharmacological control of a therapeutic that is delivered by an *in vivo* vector because it will allow titration of the circulating protein into the desired range, enable the adaptation of a therapy to fluctuating daily dosing regimes and allow reversibility of the therapy as needed (16,17).

Heterologous gene regulation strategies are also used to reprogram mammalian pathways to achieve therapeutic or bioprocess goals (9,18). Cell proliferation control is a particularly important parameter because it dominates many aspects of human disease and therapy including apoptosis (19), differentiation (20), regeneration (21) and cancer (22). In nature, molecular pathways promoting positive and negative proliferation control are carefully balanced by regulation of opposing genes (4). Overexpression of the human CKI p27^{Kip1} alone arrests mammalian cells in the G_1-phase of the cell cycle (9). However, when the same gene was combined with the growth-promoting sT gene in a dual regulated expression configuration, p27^{Kip1} expression did not induce sustained growth arrest even when sT was repressed by addition of pristinamycin (2 µg/ml). This experiment demonstrates the importance of balanced expression of genes with opposite growth effects because the residual expression of one gene may override the effect of the other. It is a major concern when implementing positive proliferation control engineering strategies in a human therapeutic context that attendant genes can be reliably silenced or their function overcome by induction of growth-arresting genes to prevent carcinogenesis. Based on such considerations, sT or other transforming genes are not ideal candidates to implement positive proliferation control in tissue engineering models. Rather, the reprogramming of quiescent cells to re-enter the cell cycle must mimic natural growth-inducing situations for the optimal integration of metabolic engineering into a complex regulation network. One approach to this was based on chemically induced dimerization [CID (23)] of signalling domains taken from endogenous erythropoietin (24), or thrombopoietin receptors (mpl) (25). Recent *in vivo* expansion of bone marrow cells engineered for CID-responsive mpl signalling did not result in leukemia in a group of five non-CID-treated control mice monitored over 1 year (26). However, truncated versions of the proto-oncogene c-mpl carried by the myeloproliferative leukemia virus (MPLV; v-mpl) have been reported to immortalize hematopoietic progenitor cells (27). Due to the fine line between successful growth-inducing engineering strategies and the risk of long-term induction of neoplastic growth any therapeutic cell expansion strategy will likely require counteracting growth-arresting molecular interventions prior to approval in human gene therapy.

p27^{Kip1} may be an ideal candidate for opposite proliferation control because the reduction of p27^{Kip1} levels is a critical event for S-phase entry during the mammalian cell cycle (11,12,28). Constitutive antisense expression of p27^{Kip1} has enabled cells to grow for several generations in medium supplemented with insulin and transferrin (12) or in medium containing low concentrations of serum (7). Furthermore, addition of antisense p27^{Kip1} oligonucleotides (coupled with TGFβ-neutralizing antibodies) induced proliferation of hematopoietic stem cells to an extent that enabled transduction by Moloney-based retroviruses, and targeted disruption of p27^{Kip1} resulted in enhanced growth of the mice and a striking enlargement of their thymus, pituitary, adrenal and gonadal organs (29–31). We have successfully used streptogramin-regulated full-length antisense expression of human p27^{Kip1} to increase proliferation of CHO cells even under optimal growth conditions while growth could be completely blocked in the G_1-phase by subsequent tetracycline-responsive expression of sense p27^{Kip1}. Dual regulated expression technology may be used as a prototype for next generation tissue engineering strategies which will likely require reprogramming of both growth characteristics and the redirection of differentiation pathways in a desired manner (32,33).

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