BMC Biotechnology

Methodology article

A gene expression system offering multiple levels of regulation: the Dual Drug Control (DDC) system

Marina Sudomoina[1], Ekaterina Latypova[2], Olga O Favorova[1], Erica A Golemis[2] and Ilya G Serebriiskii*[2]

Address: [1]Department of Molecular Biology and Biotechnology, Russian State Medical University, Moscow, Russia and [2]Division of Basic Science, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

Email: Marina Sudomoina - sudom@inbox.ru; Ekaterina Latypova - venenum_rerum@mail.ru; Olga O Favorova - olga_favorova@mail.ru; Erica A Golemis - ea_golemis@fccc.edu; Ilya G Serebriiskii* - ig_serebriiskii@fccc.edu

* Corresponding author

Published: 29 April 2004

BMC Biotechnology 2004, **4**:9

Received: 29 September 2003
Accepted: 29 April 2004

This article is available from: http://www.biomedcentral.com/1472-6750/4/9

Abstract

Background: Whether for cell culture studies of protein function, construction of mouse models to enable *in vivo* analysis of disease epidemiology, or ultimately gene therapy of human diseases, a critical enabling step is the ability to achieve finely controlled regulation of gene expression. Previous efforts to achieve this goal have explored inducible drug regulation of gene expression, and construction of synthetic promoters based on two-hybrid paradigms, among others.

Results: In this report, we describe the combination of dimerizer-regulated two-hybrid and tetracycline regulatory elements in an ordered cascade, placing expression of endpoint reporters under the control of two distinct drugs. In this Dual Drug Control (DDC) system, a first plasmid expresses fusion proteins to DBD and AD, which interact only in the presence of a small molecule dimerizer; a second plasmid encodes a cassette transcriptionally responsive to the first DBD, directing expression of the Tet-OFF protein; and a third plasmid encodes a reporter gene transcriptionally responsive to binding by Tet-OFF. We evaluate the dynamic range and specificity of this system in comparison to other available systems.

Conclusion: This study demonstrates the feasibility of combining two discrete drug-regulated expression systems in a temporally sequential cascade, without loss of dynamic range of signal induction. The efficient layering of control levels allowed by this combination of elements provides the potential for the generation of complex control circuitry that may advance ability to regulate gene expression *in vivo*.

Background

To achieve effective control of gene expression *in vivo*, higher eukaryotes generally utilize extensive promoter/enhancer/locus control regions spanning many kilobases of DNA, and encompass multiple discrete binding sites for transcription factors that combinatorially encode specificity of gene transcription. However, for studies of gene function requiring the introduction of heterologous protein into cultured cells, there is a practical limit governing the size of promoter that can be utilized, corresponding to the 2–3 kb of enhancer/promoter sequence that can be accommodated on a plasmid that also must contain other required sequence elements. Currently, many of the gene expression systems commonly utilized for studies of

protein function utilize small, virally derived enhancers (for example, from cytomegalovirus, CMV) that typically do not provide many options for calibrating the expression of encoded proteins, but rather produce constitutive high levels of protein product following introduction into cells. Although some inducible gene expression systems have been developed using natural promoter elements, disadvantages of these systems prevented them from gaining broad use. As one example, although use of the metallothionein promoter [1] allows induction of gene expression through addition of metals to cell culture, the attendant cellular stress response arising from exposure to metals can cause secondary effects complicating the interpretation of resulting data.

As an alternative approach, a number of groups have explored the possibilities of developing small artificial promoters or transcriptional regulatory systems with desirable properties for gene expression control. It has long been appreciated that it is possible to generate novel transcriptional control systems by fusing separable DNA binding domains (DBDs) and transcriptional activation domains (ADs) to achieve a desired transcriptional activation specificity [2]. The fact that it is possible to create functional transcriptional regulatory control systems even in situations where the DBD and AD are not covalently attached is the guiding principle behind technologies such as the yeast two hybrid system [3]. In adapting these concepts to the design of artificial gene expression systems, previous work by some groups has demonstrated the possibility of constructing elegant combinatorial systems incorporating feedback loops, based on the integration of two-hybrid system paradigms with diverse tissue specific promoters (e.g. [4-6]). Separately, others have shown the efficient regulation of gene expression through artificial promoters dependent on the action of small molecule modulators, including tetracycline and derivatives (reviewed in [7]); streptogramin and macrolide antibiotics, [8-10]; combined coumermycin and novobiocin systems [11]; ecdysone [12]; dimerizer molecules such as FK1012 and other rapamycin-related derivatives [13,14]. Further afield, derivatives of the "quorum-sensing" circuits of bacteria, that assess cell density to regulate interconversion between biofilms and other growth forms [15], and components of the plant phytochrome system, in which promoter activity can be regulated by light [16], have been adapted for artificial promoter construction.

Each of these systems offers specific advantages for some applications. Notably, in order to generate flexible tools for general usage in achieving fine control of gene expression, it would be useful to be able to combine elements of these different systems. However, it has not to date been clear whether these artificial transcriptional regulatory reagents are sufficiently robust to be merged into more complex regulatory cascades, particularly under the conditions of transient transfection generally preferred for rapid experimentation. This is potentially a non-trivial problem, as in increasing the number of cascade elements, more control points are introduced that might contribute to loss of efficiency, specificity, or dynamic range. Nevertheless, sequential cascades combining different groups of transcription factors, with localization and activity regulated by multiple inputs from modifying factors such as kinases, phosphatases, acetylases, and other enzymes represents a standard means of gene regulation in naturally occurring living organisms. It is likely that the complexity arising from the multiple inputs leads to more fine-tuning of output, and enables a more complex biological response. Our goals in undertaking the present study were two-fold: first, to evaluate the results of arraying two discrete, artificial transcriptional control systems in contrast to the results obtained with each system in isolation; and second, optimally, to develop a reagent system that is practically useful in achieving more fine control of gene expression.

In this report we combine a set of elements developed by various research groups with new elements constructed herein to create a complex multi-component system intended for use in transient experiments. In this system, a first plasmid expresses fusion proteins to DBD and AD, which interact contingent on the presence of a small molecule dimerizer; a second plasmid encodes a cassette transcriptionally responsive to the first DBD, directing expression of the Tet-OFF protein; and a third plasmid encodes a reporter gene transcriptionally responsive to binding by Tet-OFF. In a series of tests, we evaluate the dynamic range and specificity of this Dual Drug Control (DDC) system in contrast to simpler systems, and note issues of particular importance for future tool development. Finally, we discuss several potential applications for a DDC system.

Results and discussion

To judge the potential for development of sequential drug-regulated signaling cascades using artificial components in mammalian cells, we established the following multi-component DDC system (Figure 1). A first plasmid, (pC_4N_2-R_HS/ZF3) previously developed by Rivera et al. [17] uses a CMV promoter and an internal ribosomal entry site (IRES) to co-express a DBD (derived from the homeodomain protein ZFHD1, [18]) fused to three tandemly repeated copies of human rapamycin-binding protein FKBP12, and an AD (derived from the p65 processed peptide of the NF-κB transcription factor) fused to a second rapamycin-binding protein, FRAP. These two proteins interact solely in the presence of small non-immunosuppressive rapamycin-related dimerizers, such as AP21967 [19]. A second plasmid (pZBS-Tet-OFF, for

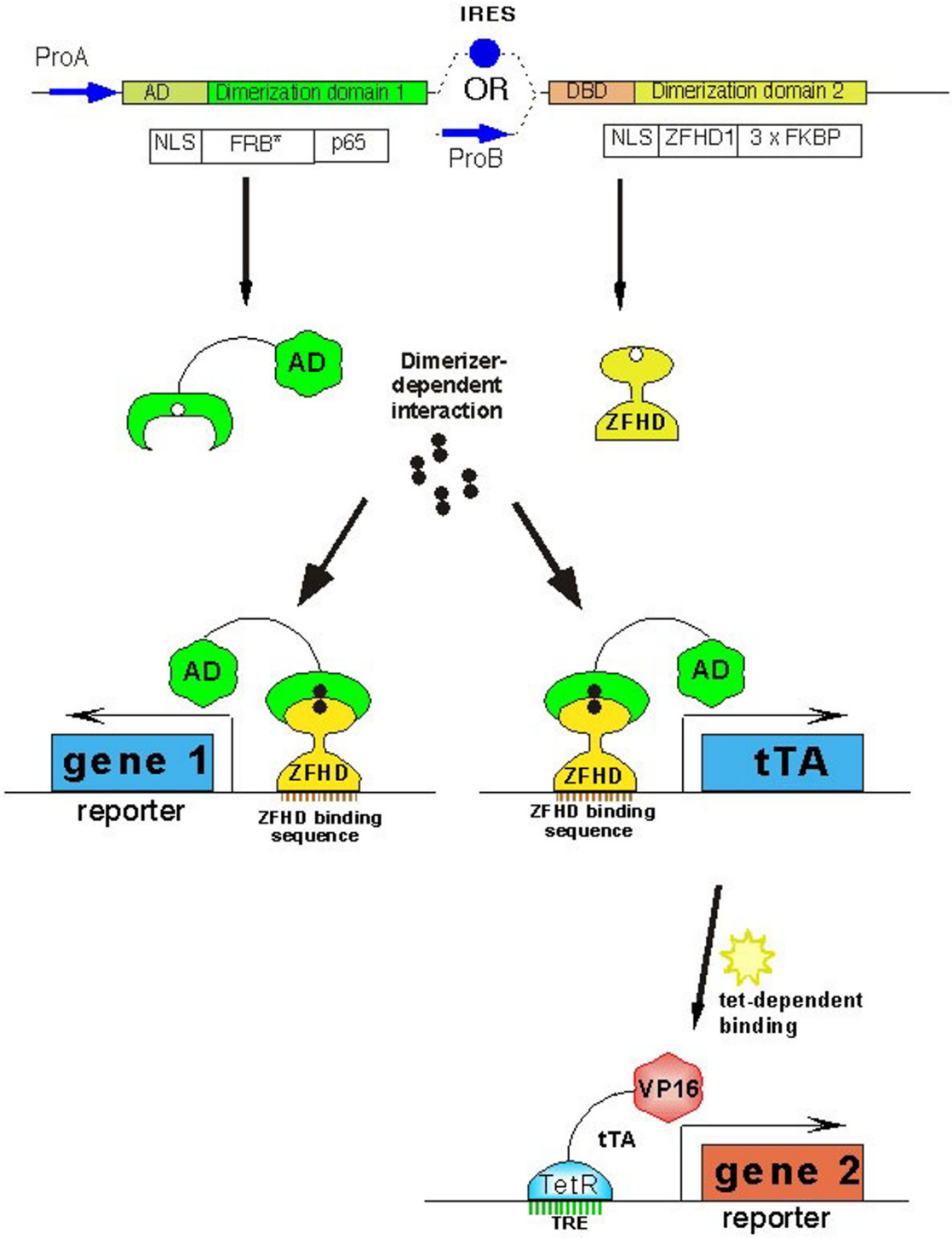

Figure 1

A. Schematic of system. DBD (ZFHD) and AD (p65) fused components are co-expressed either as a single unit from one CMV promoter using an IRES element, or are separated to two different plasmids (DBD from pAr-DBD and AD from pAr-AD). In the presence of dimerizer, these elements activate expression of a ZFHD-responsive reporter (hGH or SEAP) and the tTA protein. In the absence of tetracycline, tTA activates expression of gene 2 (SEAP or GFP).

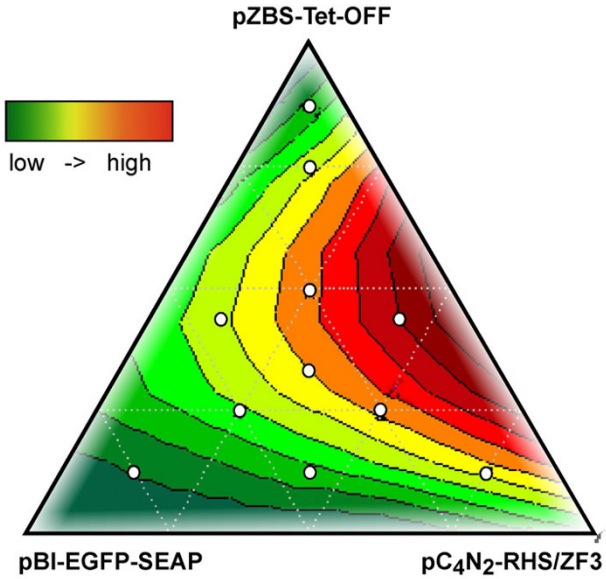

Figure 2
Optimization of induction signal. A mixture of 3 plasmids (pC_4N_2-R_HS/ZF3, pZBS-Tet-OFF, and pBI-EGFP-SEAP) was used for cell transfection; the total amount of plasmid DNA was constant at 0.5 μg per test point. As graphically represented, at each apex of the triangle shown, only one of the test plasmids is present (100% of mix), while opposite the matrix this plasmid is absent (0% of mix), with the two other plasmids present in equal proportions. White circles indicate ratios of plasmids evaluated for degree of induction of SEAP activity. Degree of induction reflects difference between growth without dimerizer and with tetracycline (OFF), versus with dimerizer and without tetracycline (ON). Based on the ON:OFF ratios obtained, the software program "Statistica" modeled the optimal response surface, shown as a contour plot on the triangle. Faded edges of the triangle represent limitations of the model, because it is meaningless whenever one of the components is absent. The optimal ratio for plasmid transfection with the DDC system was 1.5:1.5:1 (pC_4N_2-R_HS/ZF3:pZBS-Tet-OFF:pBI-EGFP-SEAP). This contrasted with a ratio of 3:1 (activator proteins:reporter) used for the original ARIAD system.

ZFHD Binding Site) contains a binding site for ZFHD-fused activators to direct transcription of the tTA gene, encoding a protein that activates transcription only in the absence of bound tetracycline [20]. Finally, a third plasmid (pBI-EGFP-SEAP) contains a binding site for tTA upstream of the SEAP gene, providing a final readout for regulation of the upstream regulatory modules.

Our first goal was to determine whether this artificial transcriptional cascade worked in a transient mode, and if so, to optimize the dynamic range of gene expression. We therefore performed a titration experiment in which we systematically varied the relative ratios of the three component plasmids transiently transfected into cells (Figure 2A). For this experiment, SEAP levels were assessed after incubation under conditions in which the reagents were predicted to be operative (with the AP21967 dimerizer, lacking tetracycline), and compared to SEAP levels under non-operative conditions (without dimerizer, plus tetracycline). These experiments first demonstrated that a robust SEAP signal was detectable under conditions of dual drug induction over a range of plasmid concentrations and allowed to determine the optimal plasmid concentrations to achieve a broad dynamic range between the induced and uninduced conditions (see legend, Figure 2). Based on this preliminary study, we selected a ratio of 5:3:2 for pC_4N_2-R_HS/ZF3: pZBS-Tet-OFF: pBI-EGFP-SEAP, respectively.

We then characterized the inductive properties of the optimized DDC system versus the Tet-OFF and dimerizer parental systems from which it was derived (Figure 3), using comparable quantities of total plasmid in each case. In these experiments, treatment of the parental Ariad dimerizer system with AP21967 (Figure 3, column A) induced activation of the ZFHD-responsive SEAP reporter pLH-Z_{12}-I-S over uninduced background by up to ~156-fold, with this large dynamic range arising in part from the extremely low background of this system in the uninduced state. By contrast, removal of tetracycline from the medium of cells containing the Tet-Off system (Figure 3, column B) induced activation of Tet-responsive- SEAP reporter over uninduced background by ~23-fold. Varying levels of dimerizer had no effect on the activity of the Tet-OFF system, and varying levels of tetracycline had no effect on the activity of the dimerizer system (results not shown). As shown, the novel DDC system combined the two requirements specified by the two different parental systems (Figure 3, column C). Activation of the SEAP reporter was only observed in the presence of dimerizer, and in the absence of tetracycline, with all other conditions showing comparable low background. The total degree of induction observed in a typical experiment was in the order of 30–40-fold, comparable to previously optimized values. Equivalent levels of induction were observed in two different cell lines, Cos7 and HeLa. Importantly, the observed degree of induction with the new system was comparable to that obtained with use of the Tet-OFF parental system, and reduced only 3–5-fold versus the ARIAD system, indicating that neither gain nor loss of signal strength was observed by the combination of the two parental systems into a two-tier DDC signaling systems. In terms of explaining the moderate reduction in dynamic range versus the ARIAD system, we believe that this is most simply attributable to the degree of basal transcriptional activity obtained from the Tet-Responsive

SEAP promoter, as similar total values of SEAP activity were obtained from DDC and SEAP systems, while reporter manifested detectable SEAP activity even in the absence of any additional components (Figure 3, column D).

We next asked if it were possible to obtain the sequential activation of different readouts from a single set of plasmids, using ordered addition of drugs. By incorporating a bi-directional promoter at the middle, ZFHD-dependent, tier of the DDC signaling cascade (see Figure 1), it should be possible to transfect a single set of plasmids into cells; induce a first gene and the tTA protein by dimerizer addition after 1–2 days; then induce a second gene by tetracycline removal. We modeled the feasibility of a two-stage induction (Figure 4). Four parallel sets of cells (groups A-D) were transfected with pC_4N_2-R_HS/ZF3 (to co-express dimerizer-dependent ZFHD- and AD fusion proteins), pZ_{12}I-hGH-2 (as a ZFHD-dependent reporter), pZBS-Tet-OFF (for ZFHD-dependent expression of tTA), and pBI-EGFP-SEAP (for tTA-dependent expression of SEAP). Group A was grown in dimerizer-/tetracycline+ medium, while groups B, C, and D were grown in dimerizer+/tetracycline+ medium. After 2 days, groups A and B were processed for analysis, while groups C and D were grown for 2 more days in dimerizer+/tetracycline+ medium, or dimerizer+/tetracycline- medium, respectively, before processing (Figure 4). As shown, as robust induction of the downstream SEAP component is observed in this two-stage induction process as was obtained in a simultaneous induction.

Finally, an advantage of the ARIAD system as developed by [14] is that the use of an IRES element to express DBD and AD fusion proteins from a single plasmid, simplifying transfections. A possible disadvantage of the use of this otherwise economical system is that it requires the expression of DBD and AD fusion proteins from the same enhancer. Some reports have suggested the combination of two different cell type- or cell cycle-restricted promoters to express DBD- and AD- fused proteins to target expression of DBD-dependent reporters to cellular compartments in which both promoters were active (reviewed in [21]): other means of using two discrete promoters to add further levels of control to a gene expression system can be readily imagined. Therefore, we next tested whether the DDC reporter system behaved comparably under the original conditions, in which an IRES element was used to coordinately express the DBD- and AD- upstream components from the CMV promoter (the pC_4N_2-R_HS/ZF3 plasmid), versus in a situation in which the expression of these components were separated to two different plasmids (the DBD-$FKBP_{12}$ protein from pAr-DBD and the AD-FRAP protein from pAr-AD) each independently under the control of the CMV promoter. While signal was still obtained under "split" conditions, the induction over baseline was very low (data not shown), such that under the best circumstances the degree of induction of the pLH-Z_{12}-I-S reporter did not exceed 20–30% that obtained with the IRES system in three different cell lines (Table 1). The reduced signal seen with the split system might be due to promoter competition (between two powerful CMV promoters, rather than one), or due to difficulties in optimizing plasmid content within cells with 4, rather than 3, plasmid components. Western analysis indicated similar expression of AD-FRAP whether expressed from an IRES or split system (results not shown). Further, generation of a split system in which one component was expressed from either the cyclin A promoter [4] or the keratin 17 promoter [22], each of which has been described as working efficiently in epithelial cell lines, was significantly less robust in inducing SEAP activity than a split CMV-only system (results not shown). For these reasons, we believe the deficiency of a split system reflects difficulty in maintaining adequate levels and ratios of 4 distinct plasmids following transfection. Given the degree of induction in a two-tier system is routinely in the order of 30–40 fold, it appeared that splitting the two components would result in too great a reduction of signal intensity to make this approach feasible, so the use of separated plasmids was not further pursued.

Conclusions

In summary, this pilot study demonstrates the feasibility of combining two discrete drug-regulated expression systems in an ordered cascade. It further demonstrates that this sequential arrangement of systems does not result in unworkable reduction in dynamic range of signal induction, and shows that a comparable level of induction is obtained using simultaneous or sequential addition of regulatory drugs. As such, it may prove to be a useful addition to the toolbox of reagents for control of gene expression in mammalian cells. Other studies in this area (for example, the work of Kramer et al., [23]), have also begun to address the sequential combination of elements derived from different artificial regulatory systems, with the goal of "fine-tuning" gene expression. This is likely to represent an important future theme in promoter design, and will involve much trial and error of many system components. Ultimately, it may also provide an informative model for analysis of elements contributing to efficient propagation of information in signal transduction cascade, as systems biology evolves as an experimental science.

Methods

Plasmids

pSEAP2-Enhancer, pRetro-Off, pBI-EGFP, and pEGFP-N1 were acquired from Clontech Labs. pC_4N_2-R_HS/ZF3, pZ_{12}I-PL-2, pLH-Z_{12}-I-S and pZ_{12}I-hGH-2 were developed

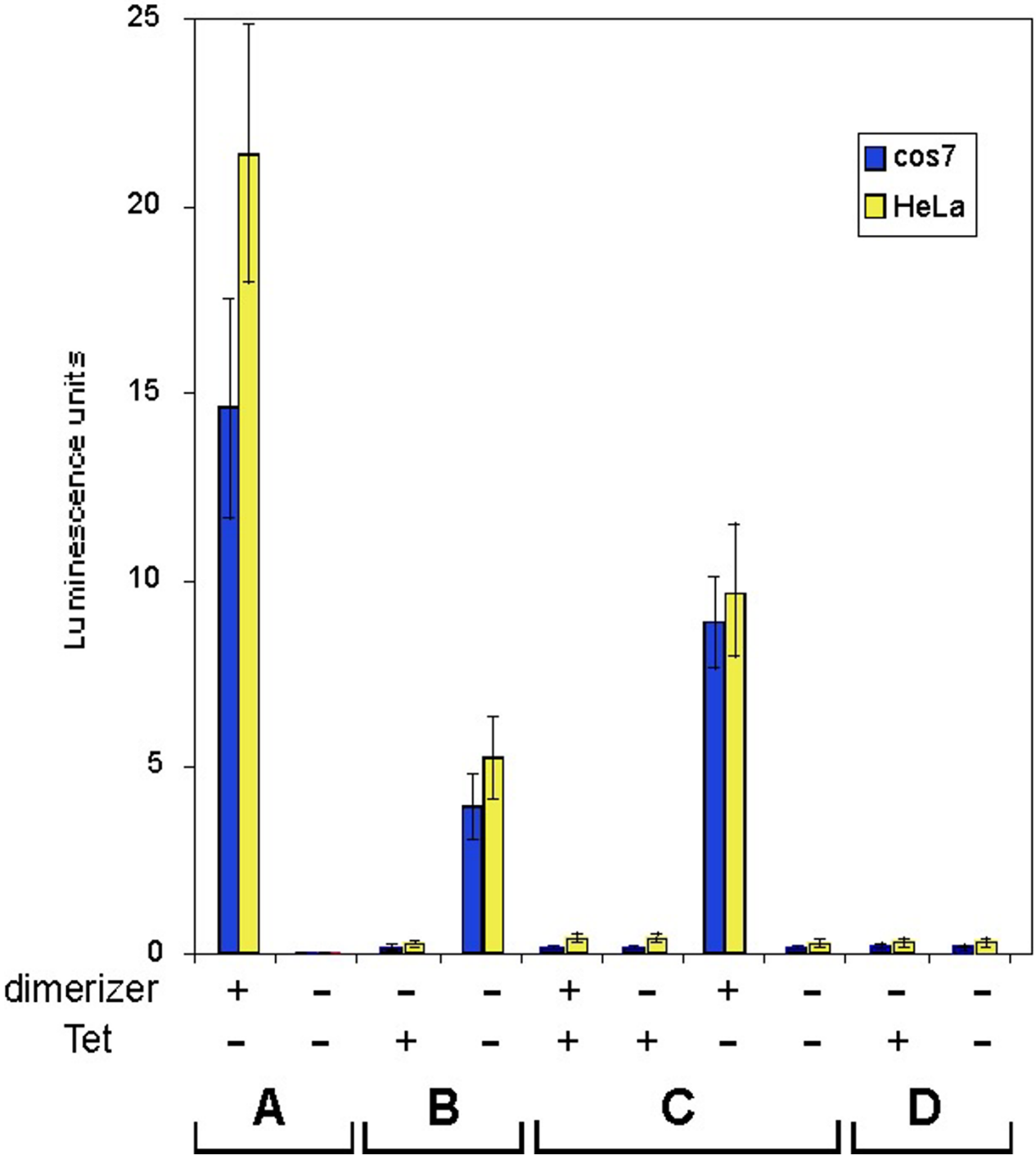

Figure 3

Demonstration of dual drug control. In Cos7 and HeLa cells, optimized ratios for the parental Ariad dimerizer System (**A**), tTA TET-OFF System (pRetro-OFF) (**B**), the merged Dual Drug Control (DDC) system (**C**), and the Tet-SEAP reporter in isolation (**D**) are evaluated for activity in the presence or absence of dimerizer and/or tetracycline. Values shown reflect luminometer readings of SEAP reporters. Blue boxes reflect values obtained in Cos7 cells; yellow boxes, in HeLa cells. Standard deviation is indicated by error bars. Each experiment was repeated at least three times.

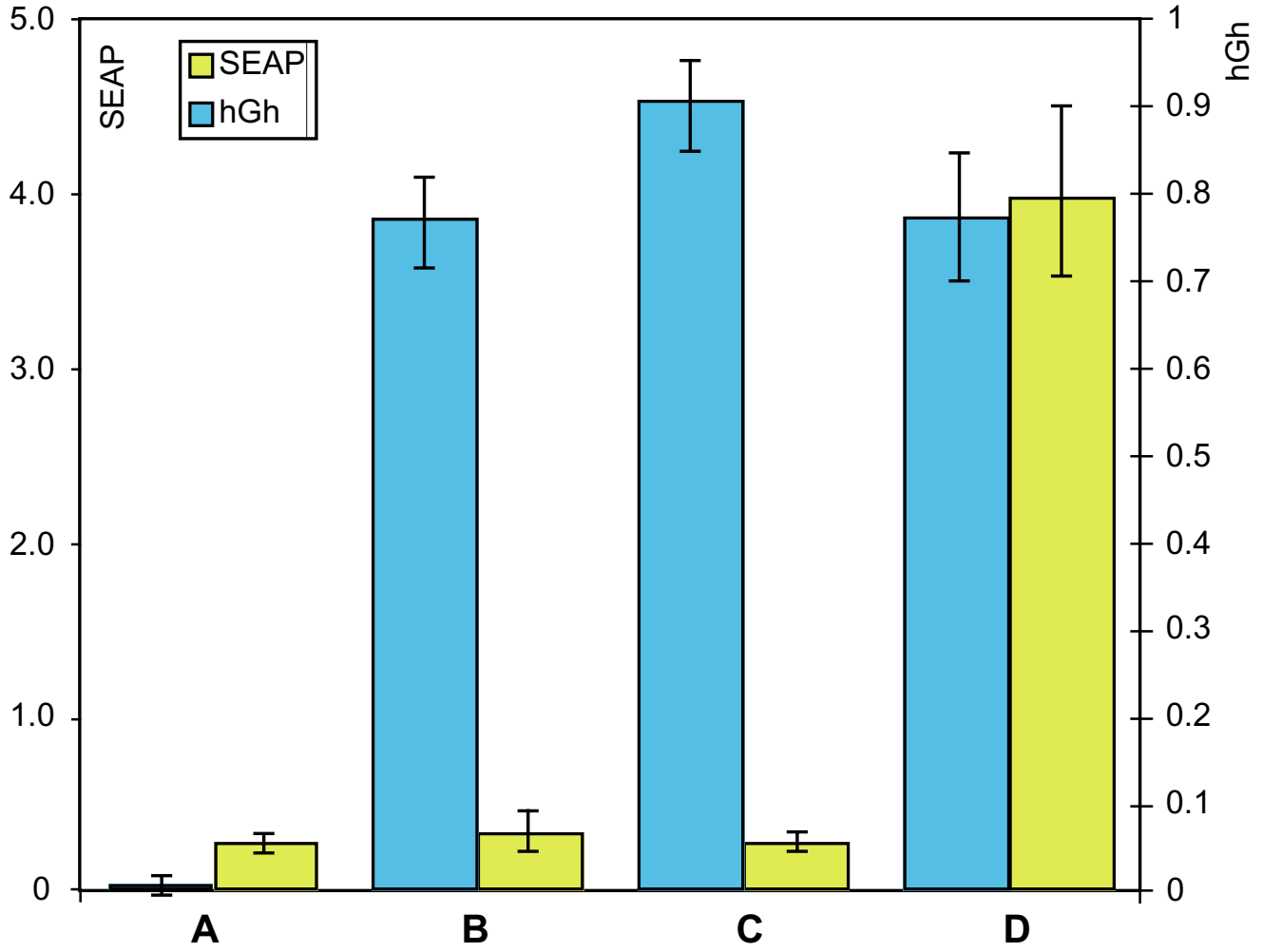

Figure 4

Inducing gene expression at two tiers. Target genes are modeled by the hGH gene (ZFHD-dependent transactivation, expression responsive to dimerizer only; scale is on the right Y-axis) and the SEAP gene (tTA-dependent transcription expression responsive to dimerizer AND tetracycline; scale is on the left Y-axis). All cells contain pC_4N_2-R_HS/ZF3, pZ_{12}I-hGH-2, pZBS-Tet-OFF, and pBI-EGFP-SEAP. See Results for details of manipulations A-D; briefly, A was grown in continuous dimerizer-/tetracycline+; B-D were initially in dimerizer+/tetracycline+. At 48 hours after transfection with plasmids, A and B were harvested, while C and D were grown for 48 more hours in dimerizer+/tetracycline+ (C) or dimerizer+/tetracycline- (D) medium before harvesting. Results of one typical experiment, with four parallel transfections for each data point, are shown. Each experiment was repeated at least three times.

Table 1: IRES versus split expression of two-hybrid components. Units shown represent fold-induction, induced over uninduced conditions, of a ZFHD-dependent SEAP reporter, in three different cell lines.

Cell line	CMV/IRES	CMV/CMV
Cos7	300	62
HeLa	335	105
A2780	222	11

by Ariad Pharmaceuticals, and are described in (the ARGENT Regulated Transcription Plasmid Kit Version 2.0, Available on-line at http://www.ariad.com/). For this work, the *Renilla* luciferase gene (SRUC3) was recloned from the plasmid pBluescript/SRUC3 [24] to replace the EGFP gene in pEGFP-N1, creating pSRUC3-N1, a reporter plasmid with expression of SRUC3 under the control of the CMV promoter. In pZBS-Tet-OFF, the pRetro-OFF vector has been modified to place the tTA (tetracycline repressor fused to viral VP16, [20]) protein under transcriptional control of 12 ZFHD1-binding sites in a minimal promoter context derived from IL2 (from pZ_{12}I-PL-2). In pBI-EGFP-SEAP, the tetracycline responsive promoter was used to bidirectionally express pEGFP and the secreted alkaline phosphatase (SEAP) gene. The plasmids pAR-DBD and pAR-AD were constructed as derivatives of pC_4N_2-R_HS/ZF3 by eliminating one of the two co-expressed components initially expressed, such that ZFHD-FKBP12 (pAR-DBD) or p65-FRAP (pAR-AD) is separately expressed under the control of the CMV promoter.

Cell culture

Cos7, HeLa, and A2780 cells were used as hosts for transfection experiments, and were cultured using standard conditions as recommended by the ATCC. Experiments for optimization of plasmid ratios were planned and analyzed using the software program STATISTICA http://www.statsoftinc.com. Transfections were performed using a *Trans*IT-LT1 reagent from Mirus. The pSRUC3-N1 plasmid was transfected together with test combinations of other plasmids (as described in Results) as a control for transfection efficiency: typically, 10% of a transfection mixture would be pSRUC3-N1, and levels of *Renilla* luciferase assessed in collected medium conditioned by transfected cells, based on standard recommended conditions [24]. In a standard experiment, transfected cells were grown in the presence or absence of 25 nM dimerizer AP21967 (Ariad Pharmaceuticals) and/or in the presence or absence of 2 μg/ml tetracycline (Sigma). Reporter activation was determined 42 – 96 hours after transfection. Comparable expression of p65 activation domain fusion proteins under induced conditions was confirmed using polyclonal antibodies directed against NFκB p65 (Geneka Biotechnology Inc.) in Western analysis.

Reporter assays

Measurement of *Renilla* luciferase activity and SEAP activity was performed in a luminometer LKB 1250. Measurement of SEAP activity in culture medium was by standard means using the chemiluminescent Great EscAPe SEAP reporter System (Clontech Labs). *Renilla* luciferase activity was measured using the *Renilla* Luciferase Assay System (Promega). In each case, cells were lysed by incubation for 15 min with *Renilla* Luciferase Assay Lysis Buffer, and the lysed samples were cleared by centrifugation for 30 sec at 14000 min^{-1} in a microcentrifuge. Data presented for SEAP were corrected based on the calculated transfection efficiency. Human growth hormone levels were assayed using an ELISA kit from Roche (# 1 585 878).

Authors' contributions

MS and EL made constructs and performed transfections, enzyme assays, and Western analysis. OF, IS and EG formulated the original idea of the project, monitored the course of the experiments, and actively participated in review of the data and organization of the manuscript. IS also took part in statistical analysis of data. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Ovarian SPORE program at Fox Chase Cancer Center, and by ACS Pilot Project Funding (to IS). We are very grateful to Tom Hamilton and Denise Connolly for useful discussions. We thank Vic Rivera for comments on the manuscript.

References

1. Brinster RL, Chen HY, Warren R, Sarthy A, Palmiter RD: **Regulation of metallothionein--thymidine kinase fusion plasmids injected into mouse eggs.** *Nature* 1982, **296:**39-42.
2. Brent R, Ptashne M: **A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor.** *Cell* 1985, **43:**729-736.
3. Fields S, Song O: **A novel genetic system to detect protein-protein interaction.** *Nature* 1989, **340:**245-246.
4. Nettelbeck DM, Jerome V, Muller R: **A dual specificity promoter system combining cell cycle-regulated and tissue-specific transcriptional control.** *Gene Ther* 1999, **6:**1276-1281.
5. Jerome V, Muller R: **Tissue-specific, cell cycle-regulated chimeric transcription factors for the targeting of gene expression to tumor cells.** *Hum Gene Ther* 1998, **9:**2653-2659.
6. Nettelbeck DM, Jerome V, Muller R: **A strategy for enhancing the transcriptional activity of weak cell type-specific promoters.** *Gene Ther* 1998, **5:**1656-1664.
7. Baron U, Bujard H: **Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances.** *Methods Enzymol* 2000, **327:**401-421.
8. Fussenegger M, Morris RP, Fux C, Rimann M, von Stockar B, Thompson CJ, Bailey JE: **Streptogramin-based gene regulation systems for mammalian cells.** *Nat Biotechnol* 2000, **18:**1203-1208.
9. Fux C, Fussenegger M: **Toward higher order control modalities in mammalian cells-independent adjustment of two different gene activities.** *Biotechnol Prog* 2003, **19:**109-120.
10. Weber W, Marty RR, Keller B, Rimann M, Kramer BP, Fussenegger M: **Versatile macrolide-responsive mammalian expression vectors for multiregulated multigene metabolic engineering.** *Biotechnol Bioeng* 2002, **80:**691-705.
11. Zhao HF, Boyd J, Jolicoeur N, Shen SH: **A coumermycin/novobiocin-regulated gene expression system.** *Hum Gene Ther* 2003, **14:**1619-1629.
12. Christopherson KS, Mark MR, Bajaj V, Godowski PJ: **Ecdysteroid-dependent regulation of genes in mammalian cells by a Drosophila ecdysone receptor and chimeric transactivators.** *Proc. Nat. Acad. Sci. USA* 1992, **89:**6314-6318.
13. Rivera VM, Clackson T, Natesan S, Pollock R, Amara JF, Keenan T, Magari SR, Phillips T, Courage NL, Cerasoli F., Jr., Holt DA, Gilman M: **A humanized system for pharmacologic control of gene expression.** *Nat Med* 1996, **2:**1028-1032.
14. Amara JF, Clackson T, Rivera VM, Guo T, Keenan T, Natesan S, Pollock R, Yang W, Courage NL, Holt DA, Gilman M: **A versatile synthetic dimerizer for the regulation of protein-protein interactions.** *Proc Natl Acad Sci U S A* 1997, **94:**10618-10623.
15. von Bodman SB, Ball JK, Faini MA, Herrera CM, Minogue TD, Urbanowski ML, Stevens AM: **The quorum sensing negative regulators EsaR and ExpR(Ecc), homologues within the LuxR**

family, retain the ability to function as activators of transcription. *J Bacteriol* 2003, **185:**7001-7007.
16. Shimizu-Sato S, Huq E, Tepperman JM, Quail PH: **A light-switchable gene promoter system.** *Nat Biotechnol* 2002, **20:**1041-1044.
17. Rivera VM, Ye X, Courage NL, Sachar J, Cerasoli F., Jr., Wilson JM, Gilman M: **Long-term regulated expression of growth hormone in mice after intramuscular gene transfer.** *Proc Natl Acad Sci U S A* 1999, **96:**8657-8662.
18. Pomerantz JL, Sharp PA, Pabo CO: **Structure-based design of transcription factors.** *Science* 1995, **267:**93-96.
19. Chong H, Ruchatz A, Clackson T, Rivera VM, Vile RG: **A system for small-molecule control of conditionally replication-competent adenoviral vectors.** *Mol. Ther.* 2002, **5:**195-203.
20. Gossen M, Bujard H: **Tight control of gene expression in mammalian cells by tetracycline-responsive promoters.** *Proc. Nat. Acad. Sci. USA* 1992, **89:**5547-5551.
21. Nettelbeck DM, Jerome V, Muller R: **Gene therapy: designer promoters for tumour targeting.** *Trends Genet* 2000, **16:**174-181.
22. McGowan KM, Coulombe PA: **Onset of keratin 17 expression coincides with the definition of major epithelial lineages during skin development.** *J Cell Biol* 1998, **143:**469-486.
23. Kramer BP, Weber W, Fussenegger M: **Artificial regulatory networks and cascades for discrete multilevel transgene control in mammalian cells.** *Biotechnol Bioeng* 2003, **83:**810-820.
24. Liu J, Escher A: **Improved assay sensitivity of an engineered secreted *Renilla* luciferase.** *Gene* 1999, **237:**153-159.