Abstract

Keywords: Ecdysone inducible system, Tetracycline regulatory expression system, and LacSwitch expression system, Regulatory expression system

Regulatory expression systems, such as tetracycline repressible system, ecdysone inducible system, and Lac operon inducible system, have been proved of tremendous value for researchers wishing to understand the functional significance of their genes of interest. In the post-genomic era, tight and inducible expression system is of extreme importance in the identification of molecular targets genuine for disease pathogenesis and drug development. In this proposal, a simple and efficient strategy is outlined to replace the traditional individual clone-based screening of generating regulatory expression system. Taking advantage of a GFP reporter construct under ecdysone responsive element and FACSorting based upon a simplistic positive and negative selection scheme, we had been able to isolate population of HEK293 and MDCK cells tightly regulated by ecdysone in term of the expression activity of exogenously introduced gene. Our preliminary data also showed similar strategy could be exploited to set up tetracycline inducible system. An example is provided to demonstrate how a dual regulatory expression system, which conjugates ecdysone inducible and tetracycline regulated systems, could be used to study the interplay of two closely related signaling molecules.

A major challenge for biomedical researchers in the post-genomic era is to identify which genes, among thousands of candidates found by methods like library substraction, differential display, and DNA microarray, are truly important in disease pathogenesis. Although the above molecular genetic tools are powerful to pick up potential candidates in term of their differential expression levels under different physiological or pathological conditions, whether they are directly linked to pathogenesis usually awaits further functional assays in a cellular or organismal context. One of the most powerful ways of studying gene functions is to analyze the phenotypic changes when the activity of the gene of interest is varied in a precisely controled manner. Fine-tuning of gene activity is of particular importance for metabolic pathway or developmental switching when quantitative parameters usually define ultimately different outcomes. Furthermore, if the genes of interest are potentially toxic while overexpressed, such as those involved in the cell death signaling, there would be tremendous difficulties in generating useful stable cell clones. Regulated gene expression system is thus an appealing solution to these problems. However, the earlier version of regulatory expression systems, such as those exploiting heat shock, heavy metal, and steroid hormone promoters, are flawed because of the leakiness of these promoter elements in controling the gene expression and also because the inducers (heat, heavy metals, and steroid hormone) by themselves would activate endogenous genes to confound the experimental results[1]. Therefore, extensive application of these systems was limited. Recently, several newer version of regulatory expression systems became available, such as tetracycline repressible system, ecdysone inducible system, and Lac operon inducible system [2,3,4] (please refer also to appendix 1 and 2). These expression systems share in common the regulatability of the expression of exogenous genes, and offer several benefits over the traditional expression system based on constitutively active promoter: (1) Inducible expression of exogenous genes, and therefore minimize the possibility of selecting secondary mutation during the course of selecting stable clones. (2) Tightly regulated expression of exogenous genes allows physiological range of protein expression which prevents cellular toxicity potentially elicited by uncontroled overexpression of genes with pleiotropic effects. (3) A perfectly matched control cell population when the exogenous gene is either not induced or suppressed.

These regulatory expression systems are ideal tools for examining the effects of expressing the genes on cell behaviors, whereas it is not straightforward to create these inducible expression systems in every kind of cell line people usually use.

In order to exercise the powerfulness of these gene expression system, one must first transplant the regulatory system which functions well in either prokaryotic or invertebral system into higher animal cells. This process usually involves intensive cloning, cell culturing, and testing procedures, and are very time-consuming. Because of the effort required for establishing the regulatory expression system, large scale screening could not be easily performed and this makes a successful outcome can not be surely guaranteed. Although it is desirable to use inducible gene expression system for studying gene functions, it takes lengthy effort to generate inducible gene expression system by conventional strategy. Our goal is to generate inducible gene expression system in a simplistic way without going through the tedious single clone selection procedure. We would briefly describe the strategy we used in making ecdysone inducible expression system in the following section.

The first steps, transfecting cell lines with the plasmids expressing the regulatory proteins (tTA, in tetracycline repressible system, and heterodimeric ecdysone receptor in ecdysone inducible system), are not different between the traditional way and the new strategy described here (please refer to appendix 3). A plasmid encoding the regulatory protein (tetracycline-regulated trans-activator, tTA, in tetracycline repressible system, and heterodimeric ecdysone receptor in ecdysone inducible system) is stably transfected into the cell lines. These regulatory plasmids are driven by constitutively active viral promoter to ensure adequate expression of the regulatory proteins. After transfection, only cells uptaking the plasmids would express the drug selectable markers (G418 resistance for pTet-ON/OFF in tetracycline repressible systemor, and zeocin resistance for pVgRXR in case of ecdysone inducible system) and survive the drug selection. Only a few of those cells uptaking the plasmids would eventually integrate the exogenous DNA into their chromosomes; most of the uptaken plasmids are in episomal forms, and would therefore be cleared out of the cells during subsequent cell division. Finally, a population of candidate clones are enriched during the selection process, and only a certain portion of these stable clones could express the regulatory proteins in a desirable way. **In the conventional way**, the candidate clones

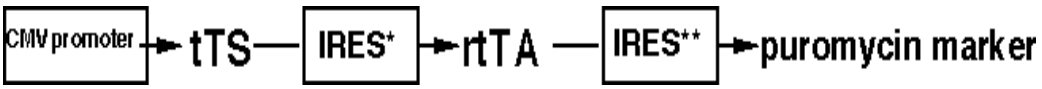
are individually expanded and tested for their inducibility, usually by luciferase reporter assay under inducible and non-inducible condition, so as to further pick up the clones possessing the optimal regulated characteristics; namely, maximal reporter activity under induced condition while minimal reporter activity under non-induced condition (please refer to appendix 4). This is the critical step in generating a regulatory expression system which demands most time and energy.

In contrast to the traditional way of selecting optimal responder using individual cloning and luciferase reporter assay, **we propose a new strategy** here. Instead of isolating and maintaining the surviving candidate clones separately, we simply trypsinize those surviving candidates after zeocin selection (zeocin resistance marker is confered by pVgRXR, the plasmids expressing the heterodimeric ecdysone receptors) and pool them all together awaiting further selection by FACS. A reporter plasmid, pIND-GFP is constructed by cloning GFP under the control of ecdysone responsive element. Then, this plasmid is transfected into the population of candidate clones which are pooled during the drug selection procedure. The responsive clones could be distinguished from the non-responsive clones simply by the brightness of GFP fluorescence under inducible condition (please refer to appendix 4), and could readily be sorted by FACS (**Positive Selection**). After several rounds of positive selection, the candidate population of cells would again be transfected with the same GFP reporter plasmid, but this time those cells with leaky expression characteristic would be sorted out by FACS under non-inducible condition (**Negative Selection**) (please refer to appendix 5).

While we had success in using the above mentioned strategy to efficiently set ecdysone inducible expression system in MDCK and HEK 293 cell lines, our initial attempt to apply similar strategy to set up tetracycline regulatory system was hampered by lack of regulatability of tetracycline responsive promoter (TRE promoter). We found the TRE promoter, which is composed by a minimal CMV promoter and seven tandem repeats of Tet operator sequence, is very active when transiently transfected into cell line. Even without the presence of regulatory proteins (tTA in Tet-OFF system or rtTA in Tet-ON system), this promoter could be activated very efficiently. The explanation for such a lack of regulated property is because the promoter, when transiently transfected into cells, is in an episomal

status, and there is no so-called chromosome inhibitory effect to govern the regulatability of the promoter. Therefore, the minimal CMV promoter in the TRE promoter could then attract transcription machinery to install gene expression even when tTA or rtTA is not binding to the promoter. To circumvent this problem, we decide to complement the Tet-ON system with a tetracycline regulated gene silencing system, as suggested by the inventor of Tet system, Dr. Hermann Bujard. tTS^{Kid-1} is a fusion protein constructed by fusing TetR (tetracycline regulated repressor), nuclear localization signal (NLS) of SV40 Tag, and a 61-amino acid KRAB domain of human kidney protein Kid-1.

This fusion protein could be combined with rtTA (regulatory protein for Tet-ON system) to provide ideal inducibility of TRE promoter for gene expression[5] (please refer to appendix 6). Our preliminary data showed it indeed could work and we are now trying to establish cell lines which express both rtTA and tTS in adequate level. Once we finish the making of such cell liens, we are going to subject them to the FACS-based positive and negative selection in order to enrich the clones which possessing optimal inducibility. Our stragety is to make use of two different IRESs (internal ribosomal entry sites) for constructing a plasmid expressing a tri-cistronic transcript.



The logical basis behind the design of this construct is as follows:

The two IRESs (IRES* and IRES**) are of different capabilities to initiate protein translation from downstream AUG start codon. IRES** is an attenuated IRES which makes puromycin selection favorable for a higher expression of genes placed upstream of IRES**. Therefore, clones surviving puromycin selection would very likely express high level of both tTS and rtTA. The expression of rtTA is coupled to the expression of tTS by an competent IRES. However, we deliberately place rtTA after tTS with the wish that every single round of positive selection by our FACSorting based enrichment strategy could not only pick up high expresser of rtTA, but also pick up high level expresser of tTS because the translation of rtTA

follows that of tTS, and both tTS and rtTA are from the same RNA transcript. Although we haven't finished making a stable cell line expressing such a tri-cistronic RNA transcript, the preliminary data show it is at least worth trying.

While we were making the Tet-ON regulated expression system, we also tried to explore the possibility of combining tetracycline regulatory expressiong system and ecdysone inducible system for studying complicated biological events. We are interested in a special form of detachment-induced apoptosis, anoikis[6]. Epithelial cells, endothelial cells, muscle cells, and perhaps many other cell types undergo programed cell death when they are deprived of the contact with extracellular matrix, which presumably interact through integrin complex to provide important cues for cellular survival and differentiation. Apoptosis induced by disruption of the interactions between normal epithelial cells and extracellular matrix has been rhetorically named as "anoikis". Anoikis is regulated by many factors, including cell-substratum, cell-cell interaction, and cytoskeleton organization. Rho family small GTPases (RhoA/Rac1/Cdc42) are major players controling all these processes[7]. Thus, it is very likely Rho family proteins regulate detachment-induced anoikis. In order to demonstrate how a dual-regulatory cell line could be used for studying anoikis, we took advantage of an MDCK cell line already expressing dominant negative Rac1 (Rac1N17) under tetracycline repressible system[8-12], and superimposed this cell line with ecdysone inducible system to express constitutively active Cdc42 (Cdc42V12). We demonstrated in this pilot study that the ecdysone inducible system could be combined with tetracycline regulated system to study the interplay of two closely related small GTPases, Cdc42 and Rac1, and their effects on detachment induced apoptosis. In this dual regulated expression system, mutant Cdc42 and Rac1 genes were inducibly expressed under the control of ecdysone and tetracycline respectively. Their effects on anoikis could then be compared directly in one single cell line in an experimentally defined way.

In summary, we are now able to exploit a simple FACS-based scheme in setting up ecdysone inducible expression system in MDCK and HEK293 cells. We are confident similar success could be expected when the strategy is applied to other cell lnes. We also tried to use the same kind of approach in establishing Tetracycline regulated expression system, but were hamper by some difficulty in getting rid of background problem. However, we are now trying to suppress the background problem by co-expressing tetracycline-regulated transcriptional activator and silencer in one single cell line. In the meantime, we have also made a cell line expressing two closely related small GTPases independently under ecydsone inducible and tetracycline repressible systems, and demonstrated indeed a dual regulatory cell expression system could be used in dissecting the signal controling anoikis. We wish in the future this system could be used in conjunction with DNA microarray technique to explore the functions of genes identified by DNA microarray method, and further used to pick up potentially interacting genes.

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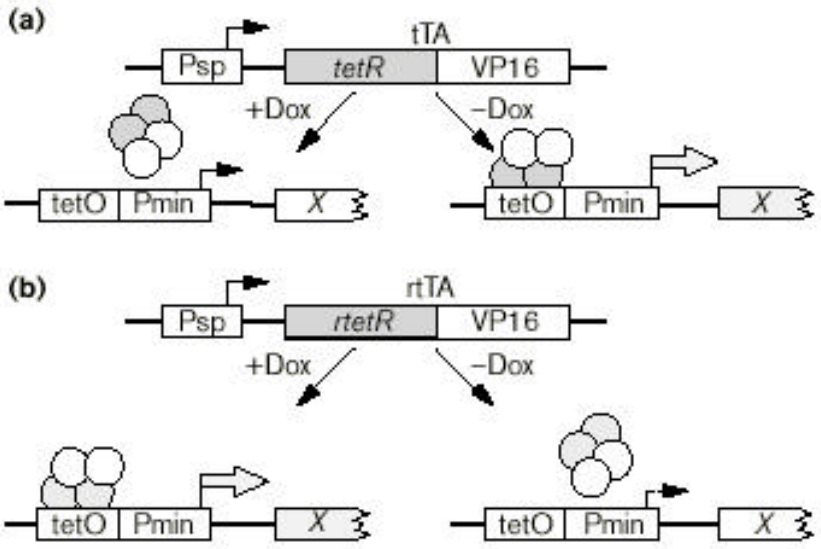
How Tetracyline repressible system works

The Tet Expression System is based on two regulatory elements derived from the tetracycline-resistance operon of the E. coli Tn10 transposon—the tetracycline repressor protein (TetR) and the tetracycline operator sequence (tetO) to which TetR binds. The gene to be expressed is cloned into the pTRE "response" plasmid which features a compound promoter consisting of seven copies of tetO (TRE), and a minimal CMV promoter.

The "response" plasmid is controled by another "regulator" plasmid which is a fusion of the wild-type TetR to the VP16 activation domain (AD) of herpes simplex virus; so called tTA standing for tetracycline regulated transactivator. tTA binds the tetO sequences which brings the VP16 activation domain into close proximity with the TRE and thereby activates transcription in the absence of Tc. Thus, as Tc is added to the culture medium, transcription is turned off in a dose-dependent manner.

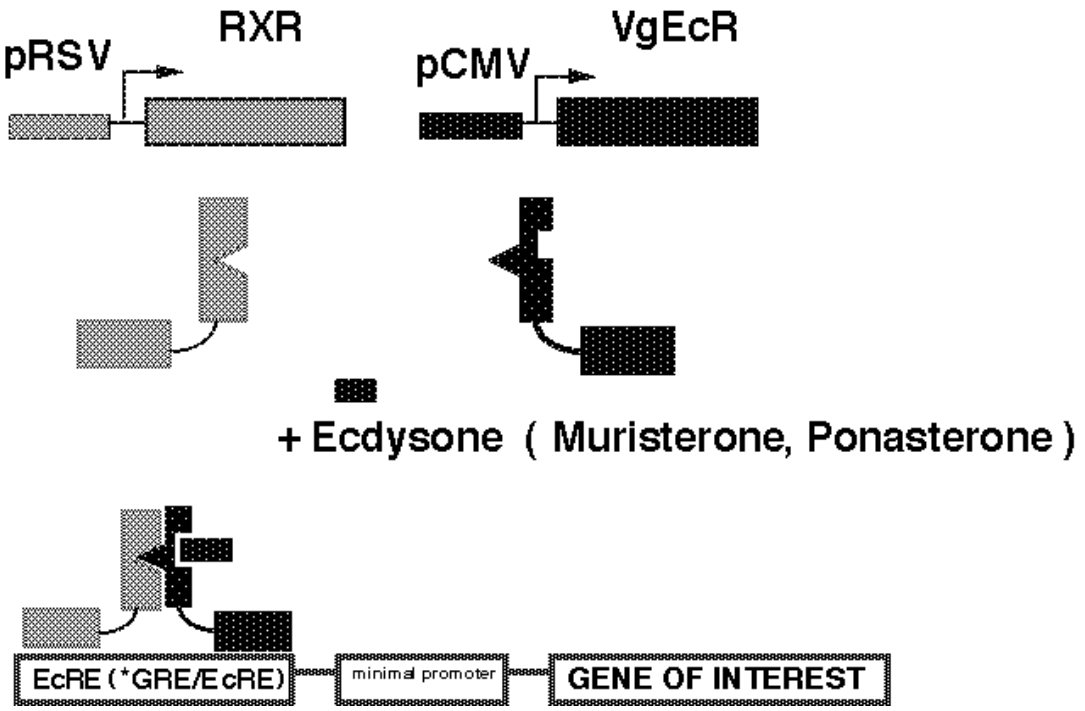
The Tet-On System is based on the "reverse" TetR (rTetR), which differs from TetR by four amino acid changes. When fused to the VP16 AD, rTetR creates a "reverse" tTA (rtTA) that activates transcription in the presence of Dox.



Appendix 1

How Ecdysone inducible system works

Ecdysone regulates Drosophila metamorphosis by binding to its receptor and transactivate genes which are responsible for the molting process. Ecdysone inducible expression system takes advantage of the fact that ecdysone heterodimeric receptor would dimerize, translocate from cytosol into nucleus, and transactivate genes located downstream to ecdysone responsive element only in the presence of its cognate ligand, ecdysone. Therefore, a mammalian cell line is firstly made to express ecdysone receptor (commercial vector expressing the heterodimeric receptor, RXR and VgEcR under RSV and CMV promoters is available). Then, your gene of interest is cloned into an expression plasmid (called pIND by the manufacturer) and placed downstream to the ecdysone responsive promoter. Finally, once the plasmid carrying the gene of interest is transfected into the cell line already expressing the heterodimeric ecdysone receptor, the expression of the particular gene would be tightly regulated by the presence or absence of ecdysone in the culture medium.



Appendix 2

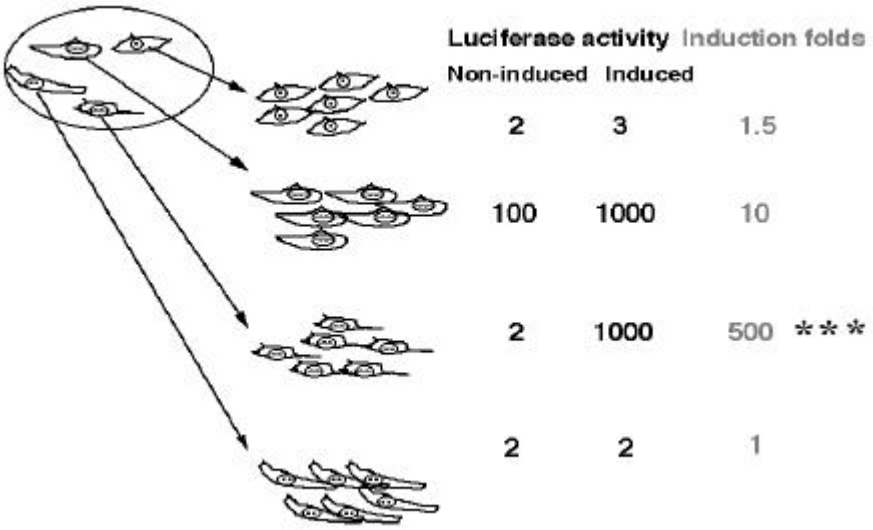
Transfecting plasmids expressing regulatory proteins into cell lines

During the first step of establishing cell lines possessing regulatory expression characteristics, plasmids expressing regulatory proteins (tetracycline-regulated trans-activator, tTA, in tetracycline repressible system, and heterodimeric ecdysone receptor in ecdysone inducible system) are transfected into the cell lines. Because the plasmids also carry drug selectable markers, the cells uptaking the plasmids could be selected in the presence of cytotoxic drugs.

Appendix 3

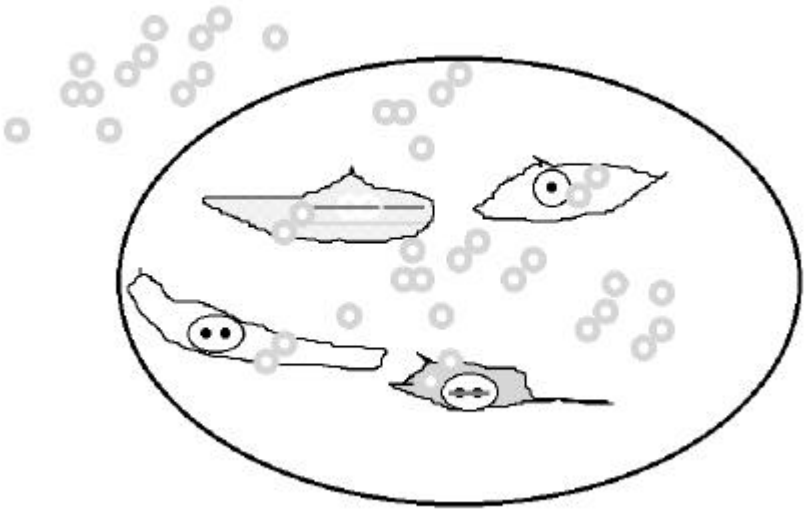
Tradition Way of Making Ecdysone Inducible Cell Line

After selecting the candidate clones by drug resistance, the individual clones are expanded and tested for their inducibility, usually by luciferase reporter assay under inducible and non-inducible condition.



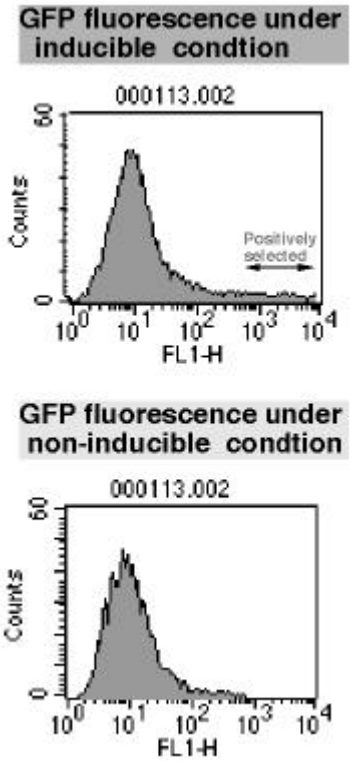
Selecting Ecdysone Inducible Cell Line by Mass Cell Culture and GFP Reporter Plasmid

A reporter plasmid is constructed by cloning GFP under the control of ecdysone responsive element. Then, this plasmid is transfected into the pooled population of candidate clones. The responsive clones could be distinguished from the non-responsive clones by the brightness of GFP fluorescence under inducible condition, and could be sorted by FACS (**Positive Selection**).



Appendix 4

How the positive and negative FACS selection work



For each round of **positive selection**, a GFP reporter plasmid is transiently transfected into the candidate population pool, and the transfected cell population was cultured in the absence or presence of ecdysone analoque, Ponasterone. Since the GFP reporter is constructed right after an ecdysone regulatory promoter, the brightness of GFP fluorescence could be used as an indication of the inducibility of the cell clones.

After substracting the clones which either do not express the GFP reporter activity or express suboptimal reporter activity in a leaky way under non-inducible condition, the true responders would be collected by FACSorting.

After several rounds of positive selection, the candidate population of cells would again be transiently transfected with the same GFP
reporter plasmid.

Appendix 5

Tet-ON system could be complemented with tetracycline regulated transcriptional silencer (tTS) to minimize leakiness of tetracycline responsive promoter

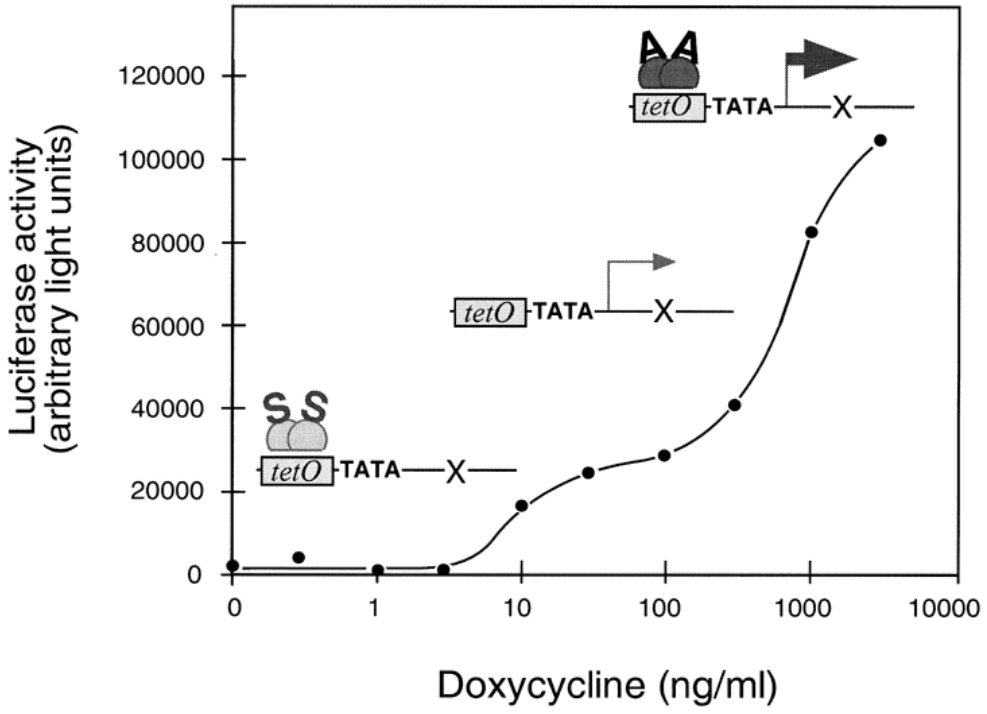
Basically, there are three players involved in making this system work:

(1) rtTA (reverse type tetracycline regulated trans-activator, which binds to Tet operator sequence, *tetO,* in the presence of tetracycline or its analoque doxycycline)

(2) tTS (classical Tet repressor fused to the transcriptional silencer, KRAB domain of Kid-1 protein, which binds to Tet operator sequence, *tetO,* in the absence of tetracycline or its analoque doxycycline)

(3) Target plasmid with a mininal CMV promoter plus seven tandem repeat of Tet operator sequence, *tetO,* followed by the reporter gene or gene of interest

By changing the concentratioin of doxycycline, the experimenter could manipulate the association status of either tTS or rtTA with *tetO,* and therefore decide whether the gene (or reporter) activity is totally shut-down (when tTS binds to *tetO* and shield it from any endogenous transcriptional machinery), or minimally expressed (when neither tTS nor rtTA binds to *tetO,* and only the endogenous transcriptional machinery acts through the minimal CMV promoter), or fully turned on (when rtTA binds to *tetO* and activate RNA transcription).



Appendix 6

Compositive images showing the fluorescence of transiently transfected GFP reporter plasmid in sequentially sorted population of MDCK cells

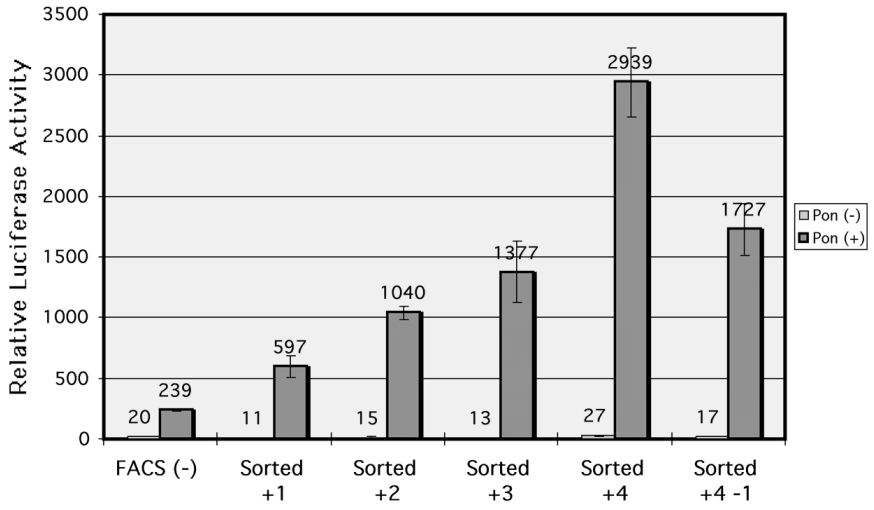
MDCK cells were stably transfected with pVgRXR (Invitrogen Co.) which expressed heterodimeric ecdysone receptors (**FACS(-)**). This population of MDCK cells were then sequentially transfected and positively selected by the green fluorescence expressed by a reporter plasmid construct (pIND-EGFP). pIND-EGFP expresses EGFP under ecdysone regulated promoter element. After being selected under inducible condition by FACSorting, cells were left in the absence of inducer; i.e., ecdysone or its analogue, ponasterone so that the fluoresence would disappear as the transiently transfected pIND-EGFP plasmids were purged from the cells. After the cells ceased emitting green fluorescence, they were sent for another round of positive selection after being transfected again with the same reporter plasmid, pIND-EGFP, in the presence of ponasterone.

Totally, there were four such rounds of positive selection. Cells were saved after each round and named according to the number of positive selection they had gone through (**Sorted +1, +2, +3,** and **+4**). As shown in the picture, the ecdysone analogue ponasterone could increasingly induce green fluorescence in pIND-EGFP transfected cells after sequential enrichment; however, there were also increasing population of green fluorescent cells under non-inducible condition. Therefore, a round of negative selection was performed by transfecting the Sorted + 4 cells with pIND-EGFP again and sorting out those leakily expressing cells under non-inducible condition. As shown in that pair of images at the bottom (**Sorted +4-1**), one round of negative selection could clean out the leakily expressing cells. Please note the total cell number in each image was revealed by Hoechst 33342 nuclear staining. The above composite image was made by transiently transfecting each collection of cells after every selection procedure at the same time with pIND-EGFP and splitting the transfected cells into two halves; one in the absence, and

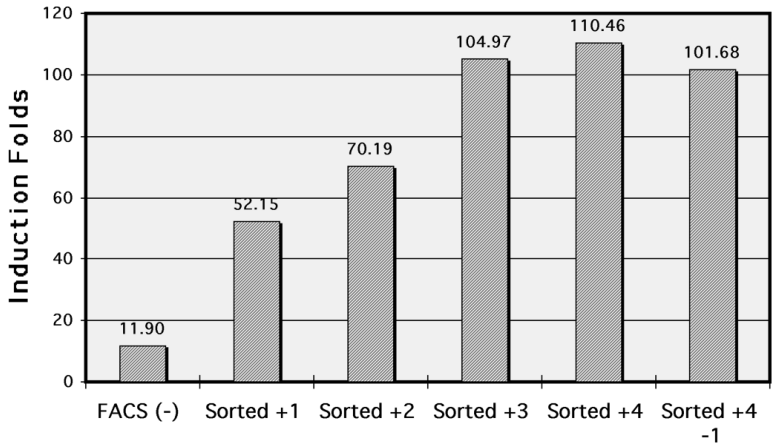
the other in the presence of inducer. This composite simulates the condition of the cells before and after each round of selection.

Quantifying ecdysone inducibility in MDCK cells

The above populations of MDCK cells were examined by luciferase activity assay for quantitative analysis of the inducibility of each collection of cells after positive and negative selection. Cells were transiently transfected with a reporter plasmid expressing luciferase under the control of ecdysone responsive element (pIND-Luc). Compatible with the previous fluorescence images, positive selection increases the expressed luciferase activity of selected cells.



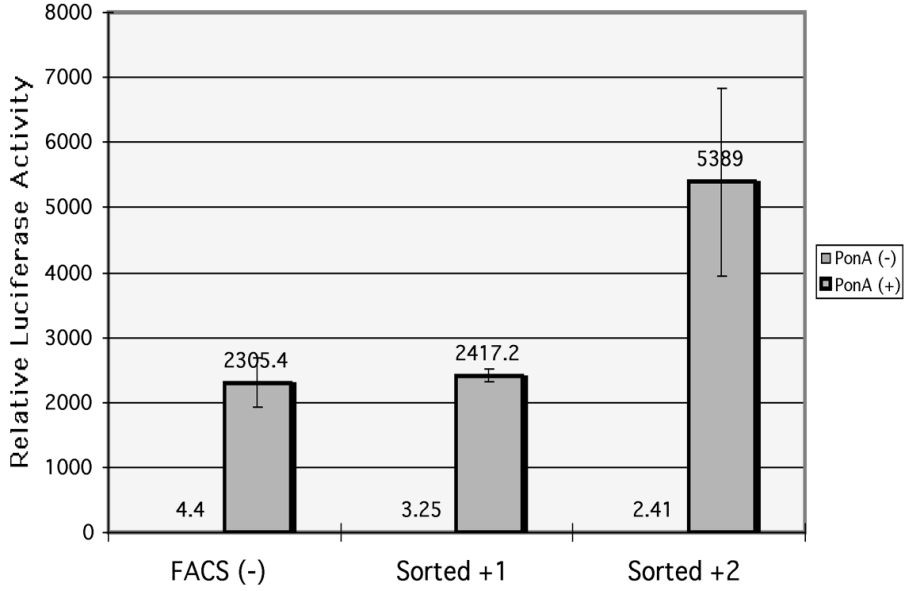
Although negative selection seemed to decrease the absolute luciferase assay value under inducible condition, the induction fold (the luciferase activity under inducible condition divided by the luciferase activity under non-inducible condition) did not further increase.

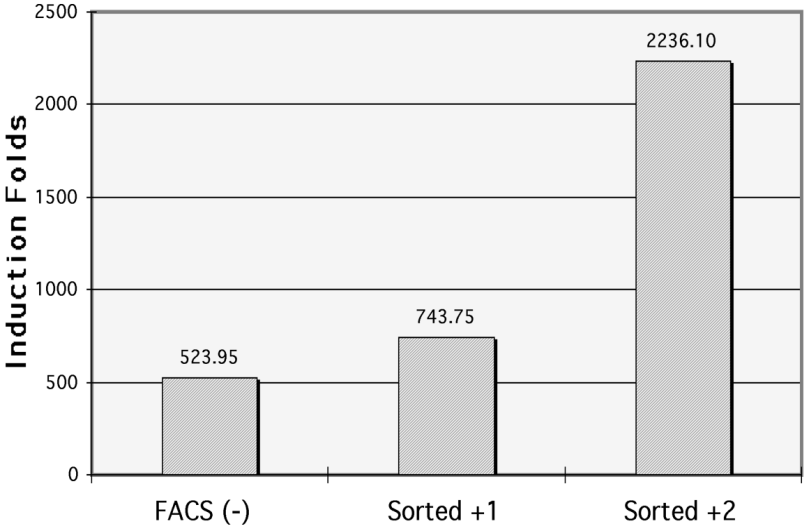


We reason this is due to concomitant decrease of the luciferase activity under both induced and non-induced condition, which reflects elimination of cells which possess leaky expression characteristics.

Quantifying ecdysone inducibility in HEK293 cells

We applied similar GFP reporter and FACSorting based strategy in HEK293 cells and had been able to establish a population of HEK293 cells responsive of ecdysone regulation. We examined the inducibility of the cells by transiently transfecting them with pIND-Luc under induced and non-induced conditions. The responsiveness is even more impressive than MDCK cells after just two rounds of positive selection. Due to the low background value under non-inducible condition, we did not perform any round of negative selection to decrease the leakiness.





Newly developed rtTA mutants could complement with Tet-TS for a better Tet-ON system

As we had mentioned in the previous section, unregulated basal transcription can be observed in Tet regulatory system if the tetracycline responsive promoter is not in a integrated status as occurs during transient transfection. Our strategy mandates the GFP reporter plasmid is expressed as an episomal form during the transient transfection step. Since there is absence of chromatin repression effect and high copy number of templates in the cells under such experimental condition , it is inevitable to note high background fluorescence even if the transfected cells are not expressing the tetracycline regulated trans-activator. To circumvent this problem, we decide to adopt a strategy proposed by Dr. Hermann Bujard, the originator of Tet regulatory system. In this system, a gene silencer protein (KRAB domain of Kid-1) is coupled to classical tet repressor to make Tet-tTS (tetracycline regulated transcriptional silencer), which could be conjugated with rtTA to accomplish high responsiveness while at the same time keeping the background level minimal.

Furthermore, we also requested from Dr. Wolfgang Hillen several newly developed rtTA mutants (S2, M2, and their corresponding humanized derivatives) which possess more favorable regulating ability than the old version of rtTA mutant (please refer to the figure at right side). According to our pilot experiment, these rtTA mutants indeed display better inducing ability when cotransfected with tet promoter regulated luciferase reporter. Our research also demonstrated Tet-TS could complement with these mutant really well

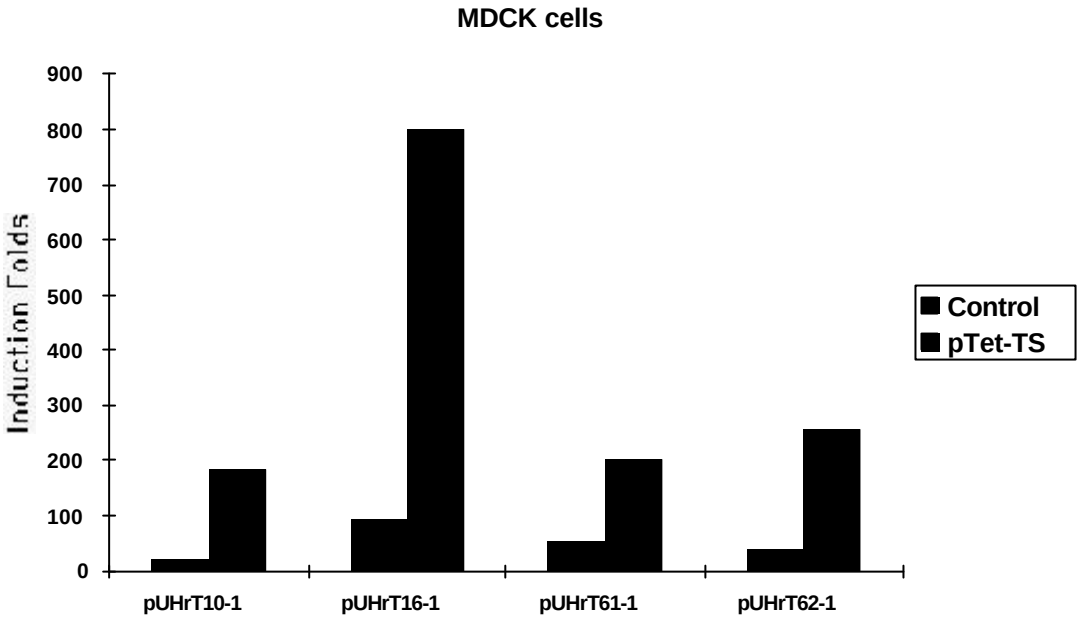
Nomenclature and mutation sites of the newly developed rtTA mutants

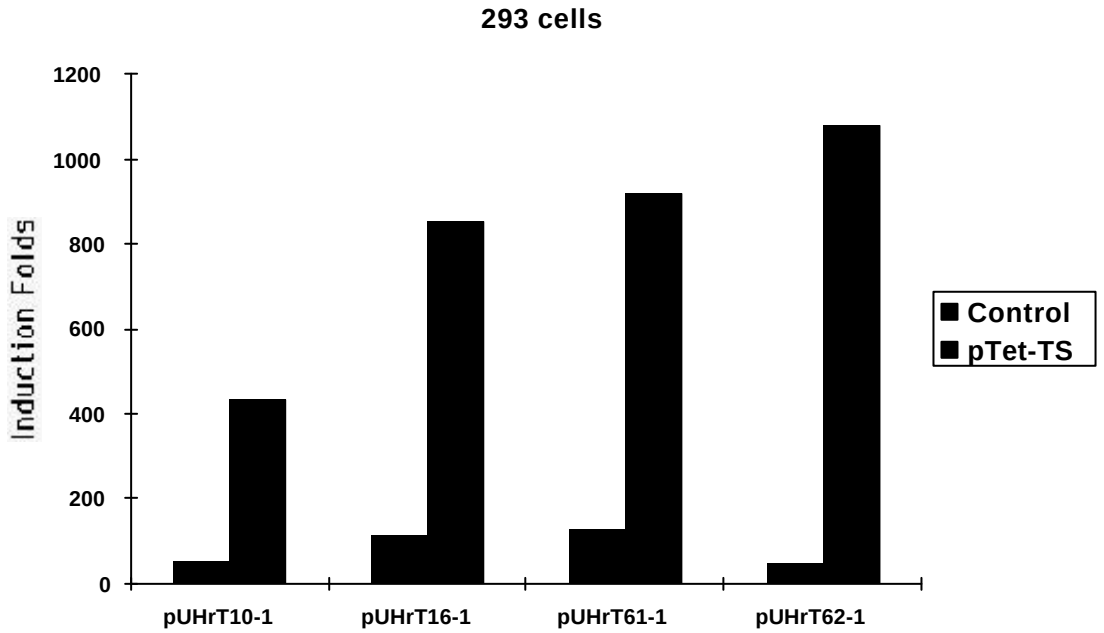
rtTA	**E^{71}->K^{71} D^{95}->N^{95} L^{101}->S^{101} G^{102}->D^{102}**
rtTA-S2	**E^{19}->G^{19} A^{56}->P^{56} D^{148}->E^{148} H^{179}->R^{179}**
rtTA-M2	**S^{12}->G^{12} E^{19}->G^{19} A^{56}->P^{56} D^{148}->E^{148} H^{179}->R^{179}**

Nomenclature of plasmids expressing the newly developed rtTA mutants

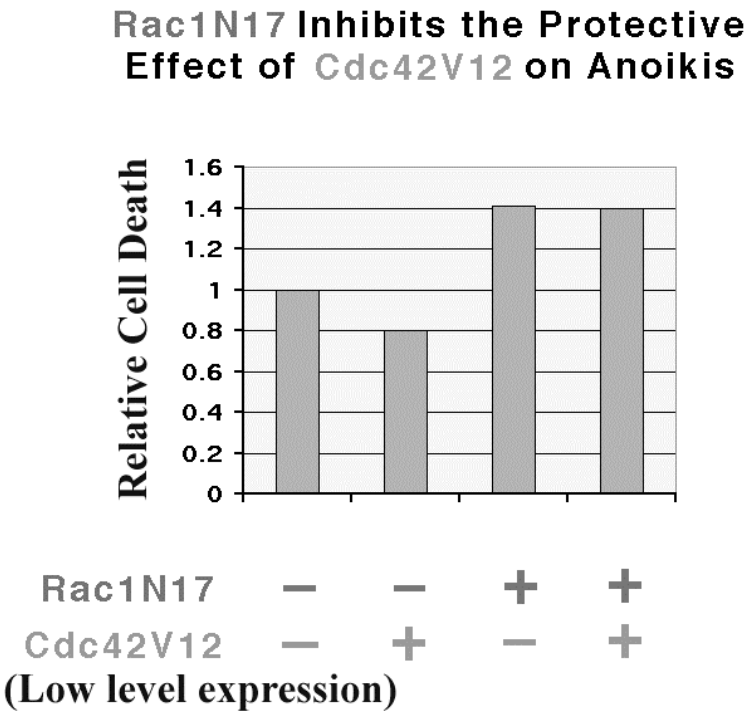
PUHrT10-1	**rtTA-S2**
PUHrT16-1	**rtTA-M2**
PUHrT61-1	**humanized rtTA-S2**
PUHrT62-1	**humanized rtTA-M2**

The above mentioned rtTA mutants were cotransfected with a control vector or a plasmid expressing Tet-TS in the presence of a reporter plasmid (luciferase gene constructed under tetracycline regulated promoter). In both MDCK and HEK293 cells, cotransfection of Tet-TS further increase the inducibility of the reporter by rtTA mutants.





While we were endeavouring to refine the strategy in order to efficiently establish ecdysone inducible and tetracycline system, we also explored the possibility of combining these two systems into a dual regulatory expression system. The Rho Family GTP-binding proteins Rac, RhoA, and Cdc42 have been shown to control distinct features of the actin cytoskeleton. Over the past several years, it has been shown that each of these GTPases plays an essential role in malignant transformation by oncogenic Ras. Rac is predominantly involved in serum-independent growth, whereas Cdc42 plays a key role in integrin signaling. We had previously made MDCK cells expressing constitutively and dominantly negative Rac1, RhoA, and Cdc42 under tetracycline repressible system. We observed distinct effects of Rac1, RhoA, and Cdc42 on detachment-induced apoptosis, anoikis; constitutively active Rac1 protects MDCK cells from anoikis, constitutively active RhoA enhances anoikis in MDCK cells, while Cdc42 has biphasic effect on anoikis (low level activation of Cdc42 protects anoikis, but high level Cdc42 activation enhances anoikis). It had also been demonstrated in Swiss 3T3 fibroblast, there is a signaling cascade from Cdc42 to Rac1, and from Rac1 to RhoA. In order to prove there is also a similar signaling pathway in MDCK regulating anoikis process, we took advantage of an MDCK cell line which expresses dominant negative Rac1 (Rac1N17) small GTPase under tetracycline repressible system, and superimposed this cell line with ecdysone inducible system to express low level constitutively active Cdc42 (Cdc42V12). When we deprived this cell line of cell substratum interaction to induce anoikis, the degree of cell death was similar to that in Rac1N17 expressing cells. This result strongly implies Rac1 plays an downstream role to Cdc42 in anoikis regulation.



<u>Specific Aim 1:</u> Generating ecdysone inducible expression system in HEK293 cells by FACSorting technique

<u>Specific Aim 2:</u> Generating ecdysone inducible expression system in 4 different epithelial cell lines (including Hela, MDCK, HaCaT, and A431)"**at the same time**

<u>Specific Aim 3:</u> A single step to generate ecdysone inducible and tetracycline dual regulatory expression systems in HEK293 cells

In our proposal, we planned to achieve the above goals in two years. Right now we have been successful in making ecdysone inducible system set up in HEK293 and MDCK cells, and are in the process of establishing similar system in Hela and HaCaT cells. Due to the fact that Tet system allows expression under transient transfection condition, our strategy needs to be modified for tetracycline inducible system. This is the main hurdle lying ahead (which also consumes most of our time and energy). However, we are quite optimistic in the result of utilizing the tetracycline regulated silencer (tTS) system in conjunciton with Tet-ON system to solve the problem. Once we demonstrate this approach is workable, we would gear up and push forward to make dual-regulatory expression not in HEK293 and MDCK cell lines, but also in other cell lines.