Orthogonal Control of Endogenous Gene Expression in Mammalian Cells Using Synthetic Ligands

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ABSTRACT: Gene switches have wide utility in synthetic biology, gene therapy, and developmental biology, and multiple orthogonal gene switches are needed to construct advanced circuitry or to control complex phenotypes. Endogenous vascular endothelial growth factor (VEGF-A) is crucial to angiogenesis, and it has been shown that multiple alternately spliced VEGF-A isoforms are necessary for proper blood vessel formation. Such a necessity limits the utility of direct transgene delivery, which can provide only one splice variant. To overcome this limitation, we constructed a gene switch that can regulate the (VEGF-A) locus in mammalian cells by combining an engineered estrogen receptor (ER) ligand-binding domain (LBD), a p65 activation domain, and an artificial zinc-finger DNA binding domain (DBD). Our gene switch is specifically and reversibly controlled by 4,4'-dyhydroxybenzil (DHB), a small molecule, non-steroid synthetic ligand, which acts orthogonally in a mammalian system. After optimization of the gene switch architecture, an endogenous VEGF-A induction ratio of >100-fold can be achieved in HEK293 cells at 1 μM DHB, which is the highest endogenous induction reported to date. In addition, induction has been shown to be reversible, repeatable, and sustainable. Another advantage is that the ligand response is tunable by varying the clonal composition of a stably integrated cell line. The integration of our findings with the technology to change ligand specificity and DNA binding specificity will provide the framework for generating a wide array of orthogonal gene switches that can control multiple genes with multiple orthogonal ligands.

Introduction
Since their inception, small-molecule controlled transcription regulators, or gene switches, have been powerful tools in many areas of biological and biomedical sciences. For example, in developmental biology, signaling pathways can be dissected through temporal suppression and expression of genes within the pathways. In gene therapy, the induction of a beneficial gene product can be regulated to fall within a therapeutic window or ceased when necessary (Buskirk and Liu, 2005; Clackson, 2000; Markoulaki et al., 2009; Zhu et al., 2002). With the advent of synthetic biology, complex gene circuits can be built from a set of well-behaved gene switches, thus creating further demand for orthogonal gene switches (Fussenegger, 2001; Weber and Fussenegger, 2009). In addition, there is a growing interest in controlling complex phenotypes, especially in mammalian cells. Controlling complex phenotypes requires the remodeling of an intricately interconnected genetic network. Due to the network’s size and complexity, many cellular phenotypes are inaccessible via the control of a single gene locus. For example, the induction of pluripotency in human somatic cells requires the simultaneous induction of at least three genes (Nakagawa et al., 2008; Takahashi et al., 2007; Yu et al., 2007). To maximize the control over the genetic network, it is highly desirable not only to control multiple genes at the same time, but also to control them using independent orthogonal ligands.
The aim of this work is to develop a framework for the engineering of orthogonal mammalian gene switches, and demonstrate its utility through the ligand-dependent induction of an endogenous gene. Compared to typical multi-copy plasmids, genes on the chromosome has a much lower copy number, and the induction of endogenous loci is often more challenging, therefore placing higher requirement on the efficiency of the design. Induction of endogenous genes is of particular interest in mammalian systems because its gene products have shown better therapeutic efficacy compared to those of an exogenous gene, possibly by providing the necessary splice variants (Rebar et al., 2002). Such functionality will also be useful to developmental biology and synthetic biology because it sometimes takes multiple splice variants working in tandem to achieve a desired biological effect (Rebar et al., 2002).

Tetracycline receptor, ecdysone receptor, chemical dimerizers, and nuclear hormone receptor are among the leading platforms for mammalian gene regulation (Clackson, 2000). In this study, we focus on the nuclear hormone receptor platform because (a) the protein can be fully humanized, which minimizes the chance of immune response, (b) the ligand specificity can be engineered and changed to create orthogonal ligand-receptor pairs, and (c) its modular design allows convenient change of its DNA binding domain to target different genes. Under the nuclear hormone receptor platform, the ligand-binding domain from a nuclear hormone receptor, for example, estrogen receptor and progesterone receptor, is fused with a zinc-finger DNA binding domain that binds to the promoter region of the target gene. Although estrogen receptor itself has slight activation ability, an activation domain, for example, VP16, VP64, or p65, is typically added to enhance its induction power (Elliston et al., 1990).

The vascular endothelial growth factor A (VEGF-A) was chosen as a model target for this study. VEGF-A is an important signaling molecule in angiogenesis, and its regulation has many therapeutic applications (Yancopoulos et al., 2000). For example, the up-regulation of VEGF-A can help new blood vessels formation as a treatment for ischemia, whereas the down-regulation of VEGF-A can restrict the growth of tumors (Bao et al., 2009; Ferrara, 2005). To target the endogenous VEGF-A loci, we have utilized VZ-8, a zinc-finger DNA binding domain (DBD) engineered by Liu et al. (2001), which recognizes a 9 bp site within the VEGF-A promoter, and has been shown to activate VEGF-A expression when combined with an activation domain.

In this study, we have combined the VZ-8 DBD, the 4S LBD, and the p65 activation domain to make a gene switch. The human p65 protein is part of the NF-κB activation complex (Schmitz and Baeuerle, 1991). It is 551 amino acids (aa) long and most of the activation activity is localized at the C-terminal region (Ballard et al., 1992). To minimize basal expression level and maximize induction level, we performed domain length optimization of the p65 activation domain.
domain as well as domain permutation and linker optimization of the overall architecture.

When transiently transformed into HEK293 cells, our construct in the absence of DHB showed no VEGF-A expression beyond the normal basal level. When induced with 1 μM DHB, a 170-fold increase in VEGF-A expression was observed. This induction was roughly twice as high as that achieved with a constitutive construct. When stably integrated into HEK293, >250-fold induction was observed with no detectable basal expression. This gene switch has among the highest performance characteristics as compared to previously reported endogenous gene induction studies. Furthermore, it provides the architecture to combine the zinc-finger DBD technology with the ligand specificity engineering technology, and brings us one step closer to the ability to control multiple genes with multiple orthogonal ligands.

**Materials and Methods**

**Construction of Gene Switch**

The p65 gene segment was PCR amplified and cloned from human cDNA and the different truncations were subsequently generated by PCR. The engineering of the 4S ligand-binding domain was described elsewhere (Chockalingam et al., 2005). The VZ8 DNA binding domain was constructed by overlap extension of DNA oligos as described elsewhere (Liu et al., 2001). The long GS linker was constructed by overlap extension PCR and the truncations subsequently generated by PCR. The different parts were assembled using unique restriction sites introduced in the PCR primers, and cloned into pCMV5 for transient expression and into pLNCX2 for stable integration. A list of plasmids used in this work can be found in the Supplementary Information.

**Luciferase Assay**

HeLa cells were grown in MEM media plus 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS; UIUC Cell Media Facility, Urbana, IL) at 37°C 5% CO₂. When cells were 80% confluent, they were trypsinized and split into 24-well plates with MEM media plus 1 mM sodium pyruvate, and 5% charcoal dextran stripped calf serum (UIUC Cell Media Facility). Cells were grown for 24 h until they were over 90% confluent and transfected using 1.5 μL lipofectamine 2000 (Invitrogen, Carlsbad, CA), 100 ng β-galactosidase expression plasmid, 690 ng luciferase reporter plasmid, and 10 ng of the relevant gene switch plasmids per well. After four hours, the media was changed and appropriate ligands added. Cells were incubated for 24 h, then lysed and assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI). Luciferase levels were normalized to β-galactosidase expression.

**ELISA Assay**

HEK293 cell line was obtained from Professor Jie Chen of University of Illinois and propagated in DMEM (UIUC Cell Media Facility) with 10% FBS at 37°C 5% CO₂. Forty-eight hours before sampling, 8 × 10⁵ cells were seeded into each well of a 24-well plate (PureCoat Amine, BD Biosciences, Bedford, MA). Thirty-six hours before sampling, cells for transient expression were transfected by FuGene HD (Promega) according to the manufacturer’s recommendation using 500 ng of the appropriate gene switch plasmids. Thirty hours before sampling, ligands were added to appropriate wells. Twenty-four hours before sampling, media was changed, and ligands re-added. After 24 hours of accumulation, the supernatant was collected and assayed in duplicate by ELISA. ELISA kit was obtained from R&D Systems (Minneapolis, MN), and performed according to the manufacturer’s recommendation.

**Retroviral Integration**

Retroviral integration was performed using retrovirus packaging system from Clontech (Mountain View, CA) according to manufacturer’s recommendation. Briefly, selected gene switches were cloned into pLNCX2 retroviral vector. The retroviral vectors were co-transformed together with pVSV-G envelope vector into the GP2-293 packaging cell line to produce retrovirus. Supernatant containing retrovirus was collected after 2 days and used to infect HEK293 cells. One day after infection, HEK293 cells were put under 1,000 μg/mL G418 selection. The resistant cells were gathered after 2 weeks of selection and subsequently used for characterization studies.

**Curve Fitting**

Curve fitting was performed using OriginPro 8.6. The clonal ligand titration curve was fitted to the Hill equation, $y = \min + (\max - \min)(x^a/(k^a + x^a))$ where $n$ is the Hill coefficient and $k$ is the $K_m$. The heterogeneous population ligand titration curve was fitted using a weighted sum of 8 Hill equations. $y = (1/\sum_i a_i) \sum_i [a_i f(\min, \max, n, k_i)]$, where $f$ is a Hill equation with $\min = 0$, $\max = 5,000$, $n = 2.3$, $k_i = [3.3 \times 10^{-6}, 1 \times 10^{-7}, 3.3 \times 10^{-7}, 1 \times 10^{-6}, 3.3 \times 10^{-6}, 1 \times 10^{-5}, 3.3 \times 10^{-5}, 1 \times 10^{-4}]$, and $a$ is the weight parameter for fitting.

**Results**

P65 Optimization

Naturally evolved activation domains, such as p65, are often composed of sub-regions that possess different activation characteristics. To find a sub-region that gives low basal level and high induction level, different truncations of p65 were fused to VZ-8 DBD and 4S LBD (Chockalingam et al., 2005), an engineered LBD sensitive to DHB, which resulted in a set of p65-VZ8-4S gene switches under the cytomegalovirus (CMV) immediate early promoter (Fig. 2). These constructs were co-transformed with a reporter plasmid VEGFp-Luc, which has a VEGF-A promoter in front of a luciferase gene, into HeLa cells, and luciferase activity was assayed 1 day after treatment with DHB ranging from 0.1 μM to 10 μM.

### Curve Fitting

- **Equation:**
  \[ y = \min + (\max - \min)(x^a/(k^a + x^a)) \]
  where 
  - $y$: Luciferase activity
  - $\min$: Minimum activity
  - $\max$: Maximum activity
  - $n$: Hill coefficient
  - $k$: Hill constant

- **Parameter:**
  - $a$: Weight parameter
  - $f$: Hill equation
  - $\min$: Minimum value
  - $\max$: Maximum value
  - $n$: Hill coefficient
  - $k$: Hill constant

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  - $f$: A Hill equation with 
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    - $\max = 5,000$, 
    - $n = 2.3$, 
    - $k_i = [3.3 \times 10^{-6}, 1 \times 10^{-7}, 3.3 \times 10^{-7}, 1 \times 10^{-6}, 3.3 \times 10^{-6}, 1 \times 10^{-5}, 3.3 \times 10^{-5}, 1 \times 10^{-4}]$, and $a$ is the weight parameter for fitting.

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after DHB induction. The region 451–551 was found to give the highest induction ratio, and therefore was used as our activation domain for the remaining experiments. We also compared the p65 activation domain with the VP64 activation domain but found that p65 worked best with our construct (Supplementary Information, SI-Figs. 1 and 2).

**Domain Permutations**

Next, we attempted to use our gene switch to induce the endogenous VEGF-A expression in HEK293 cell line. HEK293 was chosen in the induction study because of its low basal VEGF-A level, and its widely reported use in endogenous VEGF-A induction studies (Dent et al., 2007; Liu et al., 2001; Tachikawa et al., 2004). Our initial construct, p65-VZ8-4S, did not show any induction activity. We proceeded to try a set of different domain permutations as listed in Figure 2. The last two dual LBD single-chain constructs, V24P and P24V, were by far the best performing constructs, outperforming the rest by at least fivefold (Supplementary Information, SI-Fig. 3).

**Single-Chain Gene Switch Optimization**

The estrogen receptor LBD dimerizes during the process of ligand activation, and it has been shown that a single-chain estrogen receptor—comprised of two LBDs fused together—can activate gene expression by intramolecular dimerization (Beerli et al., 2000; Magzenat et al., 2008). Since it was unclear how two chimeric estrogen receptor gene switches would dimerize, we postulated that the linker length between the LBDs could affect dimerization and thus its activation characteristics. A set of single-chain gene switches from the V24P and P24V constructs was constructed by inserting between the two 4S LBDs varying lengths of GS linkers (G4S)n ranging from 20 to 110 aa long (Fig. 2). This set of gene switches was transiently expressed in HEK293 cells under the CMV promoter, and the VEGF-A concentration was assayed by ELISA 24 h after DHB induction.

As shown in Figure 3A, it was found that in the V24P construct, induction level peaked with a 60 aa linker, which offered a 20% improvement over the construct with no linker. Furthermore, the basal expression level decreased with increasing linker length, bottoming out also at 60 aa. The induction ratio for V24P-GS60 was about 170-fold. A peak induction level of 2,456 pg/mL was achieved, which was significantly higher than even the constitutive constructs V28-P65 and F435P. The basal level was around 13 pg/mL, lower than the EGFP negative control, but higher than the Gal-P65 negative control. VZ8-P65 consists of a VZ8 DBD fused directly to a p65 activation domain, and served as constitutive activator of VEGF-A, whereas F435P is another zinc-finger based constitutive activator of VEGF-A obtained from Bae et al. (2003), and served as a benchmark for the observed induction level. Gal-P65 consists of a Gal4 DBD fused directly to a p65 activation domain, and served

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**Figure 2.** Diagrammatic representations of the constructs used in this study. All constructs were cloned into pCMV5 for transient expression and pLNCX2 for stable integration. P65 is the p65 activation domain of NF-κB. VZ8 DBD is the VZ-8 zinc-finger DNA binding domain that binds within VEGF-A promoter. 4S LBD is an engineered ER ligand binding domain that is activated by DHB. A: Construct used in p65 domain length optimization. Hatched box represents variable p65 length. B: Variations of gene switch used in the initial screening to identify the best architecture. C: Linker length optimization construct based on V24P.
as a control for any non-specific induction caused by ectopic expression of p65.

The P24V construct offered a different profile of activation. While the induction level appeared independent of linker length, the basal expression level increased with increasing linker length. The strength of induction of the P24V construct was less than half that of V24P, but the basal level for P24V was only 3 pg/mL (outside of standard curve), which was comparable to the Gal-P65 negative control. The very low basal expression gave the P24V construct an induction ratio of 300-fold despite its lower peak induction level.

Time Response

The speed of activation is an important gene switch parameter. Being a constitutively expressed single-step transcriptional switch, it is expected to be slower than translational switches, but comparable to that of a Tet-On system controlling a transgene (Weber and Fussenegger, 2007). To characterize the response time of the gene switch, we added DHB to identical wells at 1-h intervals, and assayed their VEGF-A concentration at the end of 12 h (Fig. 3B). VEGF-A production was clearly detectable from 3 h onwards, and increased steadily thereafter.

Ligand Response

V24P-GS60 was chosen for further characterization because of its high induction power, and we hypothesized that the basal level could be reduced with lower gene switch expression level. For further characterization, the P24V-GS60 gene switch was integrated into the chromosome of HEK293 via retroviral integration and antibiotic selection. At first, single-clone isolation was not carried out because we wanted to see how the integrated switch behaved in aggregate, given that individual clones vary in their performances.

Compared to the plasmid version, the integrated version was slightly less sensitive to the ligand, possibly due to a lower gene switch expression level from the reduced copy number per cell. An induction level of 1,895 pg/mL was achieved at 1 μM DHB and 2,519 pg/mL was achieved at 10 μM DHB, giving an induction ratio of ~250-fold (Fig. 4A). The induction appeared to taper off at 10 μM, but cell numbers were significantly less at high inducer concentration. Therefore, the cell number was counted at the time of sample collection and normalized by the measured VEGF-A level. It was found that, after normalization to cell number, there was no attenuation of induction up to 10 μM DHB. However, increasing the ligand concentration beyond 10 μM was impractical due to severe impairment on cell growth. The growth impairment most likely came from the heavy metabolic burden under high induction, because no effect on cell growth could be observed in cells not expressing the gene switch, even after prolonged DHB and/or VEGF-A exposure. The induction was log-linear to ligand concentration over at least two orders of magnitude. This characteristic allowed the switch to be tuned by external ligand concentration and achieve induction of different strengths.

Despite its usefulness in fine-tuning gene expression, a gently sloped ligand titration curve was atypical for a 4S LBD gene switch (Chockalingam et al., 2005). Suspecting that this observation might be an aggregated behavior, 12 clonal cell
lines were isolated from the heterogeneous population, and tested for their VEGF induction level at 1 μM DHB. The induction level ranged widely from 810 to 5,100 pg/mL, and the average of the 12 clonal cell line was 2,740 pg/mL, which was close to that obtained from the whole heterogeneous population (Supplementary Information, SI-Fig. 4). When we picked the most inducible clone and performed a ligand titration, we obtained a nearly perfect sigmoidal response with a Hill’s coefficient of 2.3, and a $K_m$ of 38 nM (Fig. 4B).

Further tests on other clones reflected that the $K_m$ varies between clones (Fig. 4C).

We propose that the ligand titration curve of the heterogeneous population is a weighted sum of a series of perfectly sigmoidal curves with different $K_m$. As seen in Figure 4C, using just eight evenly spaced sigmoidal curves as the basis set, we can fit the observed heterogeneous ligand titration curve very well by assigning different weightage to them.

**Sustained Induction and Reversal**

In some applications, long-term sustained induction might be required, whereas in other applications, short intervals of repeated induction might be preferable. To characterize the long-term induction, induction reversal, and repeated induction behavior of the gene switch, the stably integrated HEK293 cells was subjected to three 16-day time courses (Fig. 5). The first set of experiments was performed using the heterogeneous population of integrants. In time course 1, the cells were induced for 2 days followed by 6 days of rest; in time course 2, the cells were induced for 4 days followed by 4 days of rest; and in time course 3, the cells were...
maintained at 1 μM DHB through the entire duration. All values were normalized to the cell number at the time of sampling.

In time course 1 (Fig. 5A), the VEGF-A production level increased quickly to about 2,700 pg/mL/24 h 1 day after ligand addition, and increased further to 3,700 pg/mL/24 h on the second day. The VEGF production decreased quickly upon ligand withdrawal, reaching basal level in 2 days. When the cells were re-induced on Day 9, a similar behavior was observed. This showed that the induction was reversible and repeatable. In time course 2 (Fig. 5B), the daily VEGF-A production level increased initially for 3 days, reaching up to 4,800 pg/mL/24 h. However, VEGF-A production dropped on the 4th day despite continued induction. The induction sensitivity was only partially recovered after 4 days of rest, and a drop in induction level was still observable on the 4th day of re-induction. During sustained induction in time course 3 (Fig. 5C), a similar trend was observed for the initial 3 days of induction. The induction level then decreased steadily from the 4th day onwards. After 15 consecutive days of induction, the VEGF-A production level dropped to near basal level.

It was found that sustained induction could only be maintained using a clonal integrant cell line, and at such a level that it does not affect cell growth. The sustained induction time course was repeated using a highly sensitive clone at 20 nM DHB, which corresponds to an induction level of about 1,000 pg/mL/24 h. At this level, the induction can be sustained for the entire duration of the time course (Fig. 5D). To demonstrate that the cells were still capable of high induction, the maximum induction of 5,500 pg/mL/24 h was elicited using 200 nM DHB on Day 1 and again on Day 9.
Localization

Because of the low VEGF-A background in our test cell line, we were unable to determine if the presence of the uninduced gene switch had any effect on the VEGF-A expression. It was plausible that the DNA binding domain could still bind to the promoter and affect native transcription. To test this possibility, a localization study of the gene switch was performed, that is, if the gene switch were mostly cytosolic when un-induced, then the likelihood of it affecting native transcription would be low.

The EGFP gene was fused to the N-terminus of our gene switch and confocal microscopy was used to visualize where the gene switches were localized in the cell (Fig. 6). To ensure the EGFP-fused gene switch would behave similarly, its induction activity was also assayed. The activity data showed that the EGFP fusion protein was active, albeit at a lower level most likely due to a reduced expression level (data not shown).

The confocal images indicated that the gene switch was mostly localized within the nucleus with and without ligand. The natural estrogen receptor is known to reside mostly in the nucleus and the presence of an engineered ER LBD alone appears sufficient to confer this localization property. For natural full length ER, the un-induced receptor is bound to heat shock protein (hsp90), which prevents dimerization and blocks the transcription regulation activity of the receptor. In contrast, it has been reported that the ER LBD alone is insufficient for binding to hsp90 (Pratt and Toft, 1997). Whether this is true for our engineered gene switch remains to be tested.

Discussion

In this report, we have demonstrated that our gene switch is able to induce endogenous VEGF expression in HEK293 cells using a non-steroid orthogonal ligand. An induction ratio of over 100-fold can be achieved at µM ligand concentration. We have also shown that the induction is fast, reversible, and sustainable as long as the cellular machinery can support it.

To highlight the performance of our gene switch, it is important to make some cross comparisons with the performance of previously reported gene switches. This is, however, difficult due to differing assay conditions. We have summarized previously reported VEGF induction values, be it inducible or constitutive, and made reasonable comparisons

Figure 6. Confocal microscopy. Fluorescent gene switch constructs are created by inserting EGFP at the N-termini of the gene switch constructs used for transient expression. The EGFP constructs are transfected into HEK293 cells grown on Ibidi poly-D-lysine µ-slide (Ibidi, Verona, WI) using FuGene HD (Promega, Madison, WI). Images are taken 24 h later using Carl Zeiss LSM 700 confocal microscope (Thornwood, NY). Nucleus stain channel shows the nuclei counter stained by Hoechst dye (Invitrogen, Carlsbad, CA). EGFP channel shows the location of the EGFP-tagged gene switches.
whenever possible (Table I). Overall, our gene switch assay has among the lowest initial cell number and among the shortest accumulation time, and yet, has the highest peak VEGF induction value. Pollock et al. (2002) normalized their VEGF assay by total cellular protein content, and is therefore not directly comparable. Fortunately, both Pollock et al. and Liu et al. have included hypoxia positive control which suggests that Pollock’s assay conditions yield values roughly 8.5 times that of Liu’s (Liu et al., 2001; Pollock et al., 2002). Liu’s and Dent’s studies share a common construct, VZ + 434-P65, and their relative value suggests that Dent’s assay yields values roughly 3.5 times that of Liu’s (Dent et al., 2007; Liu et al., 2001). Our study shares a common construct with Bae et al. (2003), F435P, which suggests that Bae’s assay yields value roughly twice ours.

Taken together, our gene switch produces the highest peak induction value as well as the highest induction ratio, even when compared with constitutive constructs. It should be noted that induction ratio is highly dependent on basal level expression, and due to the low basal level expression under non-induction condition, our low-end measurement values are often too close to the detection limit of the ELISA assay to be dependable. This is especially true in the case of an integrated gene switch in a heterogeneous population, where the measured VEGF-A value is effectively zero. In that case, we have used the ELISA detection limit (around 10 pg/mL) to calculate the induction ratio. We have excluded from comparison the clonal assays value, which has a peak of 4,588 pg/mL, because it varies between cell lines and we do not believe that it is a good representation of the gene switch’s performance.

In the presence of the uninduced gene switch, the basal VEGF-A level appears lower than the negative control. This is the most pronounced in the integrated HEK293 cell line. Since our localization study found that the gene switch resides mostly in the nucleus with and without induction, it is possible for the DBD to bind and disrupt regular transcription. However, due to the high uncertainty associated with low value measurements, we are unable to determine if the un-induced gene switch represses VEGF-A transcription.

We have shown that the induction is reversible and repeatable. However, we are unable to sustain the induction over a long period of time in a heterogeneous population. This is because cell growth is inhibited and cell death is triggered at a high induction level, most likely due to overwhelming metabolic burden. Since the heterogeneous population contains cell lines that have a wide range of ligand sensitivity, we are effectively enriching the non-secreting and low-secreting cells by killing off the highly inducible ones. In a clonal cell line, induction is sustainable at a level that does not affect cell growth. Unfortunately, this is achieved at a ligand concentration that is around the $K_m$, where the induction level is most sensitive to minute differences in ligand concentration, thus leading to large errors of measurement.

We also see important differences in the behavior of the heterogeneous and the clonal integrant populations. For example, the ligand response curve is much gentler in a heterogeneous population which allows for finer control of induction level. Theoretically, by mixing a finite set of clonal population of different ligand sensitivities, we can tune the ligand response curve of the mixed population to achieve a sensitivity we desire.

As a ligand, DHB has shown very little toxicity in multiple cell lines, with IC50 greater than 100 μM (Gonzalez, 2008). Similarly, DHB has also been found to have low systemic toxicity in mice, and intraperitoneal (IP) injection of 1 mg/kg is well tolerated. While DHB is stable in cell culture media for days, it is either rapidly cleared from or modified in mice with no detectable DHB remaining in blood 24 h after IP injection. Despite the apparent short half-life, DHB has been successfully used to trigger recombination in mice through a Cre-ER fusion protein. The recombination efficiency observed using the DHB-4S ligand-receptor pair was comparable to that of the tamoixfen-ER$^{T}$ pair (Brocard et al., 1997; Metzger et al., 2010).

The ultimate aim of the technology developed in this study is to create a platform for the engineering of orthogonal gene switches that can independently control multiple genes in mammalian systems. The LBD used in our study is responsive to DHB and more orthogonal LBD-

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Table I. Comparison of assay conditions and induction values in previously reported VEGF-A induction studies.

<table>
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<th>Assay Conditions</th>
<th>Liu et al.</th>
<th>Pollock et al.</th>
<th>Bae et al.</th>
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<td>Induction type</td>
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<tr>
<td>Reference values</td>
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<td>Hypoxia</td>
<td>F435-P</td>
<td>VZ + 434-P65</td>
<td>F435-P</td>
</tr>
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</table>

When a particular condition or construct is shared between studies, the name and value is noted under reference values.

*Not specified, assume typical working volume for the plate format.
ligand pairs can be created through the established method published earlier. In fact, the method has already been successfully applied in multiple studies, giving rise to multiple orthogonal LBD-ligand pairs (Chockalingam et al., 2005; Islam et al., 2009; McLachlan et al., 2009). In order to control multiple genes, multiple DBDs also need to be engineered. Fortunately, zinc-finger DBD technology is well established and is now even commercially available (Bae et al., 2003; Miller et al., 2007; Porteus and Carroll, 2005; Zhang et al., 2000). Looking further, TAL effector DBD is a new class of DBD that promises even better modularity than zinc-finger DBD (Miller et al., 2011; Zhang et al., 2011). However, its incorporation into the gene switch construct will likely involve redesign and optimization.

To conclude, we have reported the design and construction of a highly effective ligand-responsive artificial transcription factor whose performance tops previously reported designs. This study represents the missing link between gene targeting technology and ligand specificity engineering, thus making it possible to create multiple orthogonal gene switches under the control of multiple orthogonal ligands. This technology can be immediately useful to developmental biologists, and with further development, be useful in gene therapy and synthetic biology applications.

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References


