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Designed transcription factors as tools for therapeutics and functional genomics

Fyodor D. Urnov*, Edward J. Rebar

Sangamo Biosciences, Inc., Pt. Richmond Tech. Center, 501 Canal Blvd., Suite A100, Richmond, CA 94804, USA

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Abstract

The paucity of tools that control expression of specific genes *in vivo* represents a major limitation of functional genomics in mammals; most available small-molecule regulators of transcription—e.g. histone deacetylase inhibitors—exert pan-genomic effects. Recent developments in understanding the role of chromatin in regulating the genome, and of protein–DNA interactions have allowed the development of designed transcription factors that regulate specific genes *in vivo* (Reik *et al.*, Curr Opin Genet Dev 2002;12:233). These proteins contain two modules: (i) a zinc finger protein (ZFP)-based DNA-binding domain (DBD) designed to recognize a specific sequence (for example, a motif in the promoter of a certain gene); (ii) a functional module (for example, a transcriptional activation or repression domain). Recent data describe the use of such designed transcription factors to regulate a variety of clinically relevant gene targets in human cells: these include MDR1, erythropoietin, erbB-2 and erbB-3, VEGF, and PPARγ. In the case of VEGF (Liu *et al.*, J Biol Chem 2001;276:11323), proportional upregulation by the designed transcription factor of all three distinct splice isoforms generated by this locus was observed, illuminating the utility of endogenous gene control in therapeutic settings (proper isoform ratio is essential for the proangiogenic function of VEGF). In the case of PPARγ, use of a transcriptional repressor designed to downregulate the expression of two PPARγ isoforms allowed ''mutation-free reverse genetics'' analysis that illuminated a unique role for the PPARγ2 isoform in adipogenesis (Ren *et al.*, Genes Dev 2002;16:27). The ability to selectively activate or repress specific mammalian genes *in vivo* using designed transcription factors thus has considerable promise in clinical and in basic science settings.

Keywords: Functional genomics; Mammal; Transcription; Zinc finger protein; Theraputics

1. Introduction: the challenge of the human genome

As recently noted by Sydney Brenner, ''. . .[t]he great challenge in biological research today is how to turn data into knowledge'' [1]. This is well illustrated by the near-completed sequence of the human genome: anyone with access to the world-wide-web can retrieve an enormous amount of primary data, but such *ad libitum* access to 2.9×10^9 bp of human DNA information and the ~35,000 genes it contains does not translate into knowledge about molecular mechanisms operating in human cells, tissues, organs, or the entire body and, importantly, about their dysfunction in disease.

The best-understood eukaryote, budding yeast, has not just the smallest genome of all ''model organisms'' but also a well-developed array of reverse-genetic tools for its analysis—the often-mentioned ''awesome power of yeast genetics''—which allows systematic study of individual gene function (for example, by the EUROFAN project [2]), regulatory networks [3], binary protein–protein interactions [4], and protein complex composition [5,6]. Limitations inherent to the current toolbox of mammalian genetics make many of these experiments either very difficult or near-impossible. A major technical problem is the resistance of mammalian genomes—with the notable exception of ES cells—to both homologous and nonhomologous transgenesis [7], which makes it difficult to purposefully alter the gene expression pattern of mammalian cells.

This is an issue not only for completists in academia eager to assign function to the ~12,000 human genes that currently lack one, or to dissect the relative contribution made by closely related members of gene families to a

* Corresponding author. Tel.: +1-510-970-6000; fax: +1-510-236-8951.
E-mail address: furnov@sangamo.com (F.D. Urnov).

Abbreviations: ZFP, zinc finger protein; ES, embryonic stem; MDR, multiple drug resistance; VEGF-A, vascular endothelial growth factor A; PPARγ, peroxisome proliferator activated receptor γ.

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particular pathway—the latter can be particularly challenging, as seen, for example, in genetic analysis of the chromatin remodeling ATPases hBrm/Brg1 in mouse development [8] and human cancer [9]. Analysis of human disease etiology and the development of therapeutic agents can, in principle, benefit greatly from detailed analysis of underlying molecular pathways. The clinical efficacy of such compounds as STI571 [10], a competitive inhibitor of the causative agent of chronic myeloid leukemia, the oncogenic kinase BCR/ABL, demonstrates that informed, mechanism- and pathway-based drug design is feasible. A considerable number of human diseases—many forms of cancer, for example [11]—are disorders of transcription, and pharmacological intervention via small molecule compounds with such aberrant transcription is therapeutically effective in human patients [12,13]. A general and frequently complicating property of such compounds is the relatively broad spectrum of their action—for example, the demethylating agent 5-azacytidine does, in fact, reactivate tumor suppressor genes aberrantly silenced by methylation, but its action affects the entire genome [14].

There are several ways to more selectively alter gene expression *in vivo* (all of which face the important issue of efficient delivery to the target cell, tissue, or organ). When a cDNA encoding a gene of interest is available, its transgenesis can be used to increase mRNA levels of that gene in the cell. In addition to homologous recombination-based methods which ablate gene expression entirely (for example, [15]), the lowering of mRNA levels can be accomplished via the use of antisense reagents [16], ribozymes, or RNAi [17]. In specific settings, these strategies can be quite effective.

A conceptually different approach to selectively altering gene expression involves the direct transcriptional regulation of the target endogenous locus. Several strategies have been described for this purpose. For example, selective gene regulation has been achieved by mutating existing transcription factors to create dominant negative allelic forms. A variety of technologies have also been described for the design of synthetic DNA-binding molecules—such as polyamides or peptide–nucleic acids—with pre-determined DNA binding specificities [18–20]. By targeting such molecules to regulatory sequences, selective gene control may be achieved via competitive inhibition of binding of endogenous transcription factors.

A third, and perhaps more powerful, strategy for achieving experimenter-controlled transcriptional regulation involves the design of proteins with novel DNA-binding specificities [21,22]. The chief advantage of this approach is its versatility. In principle, such designed proteins offers the possibility of targeting any functional domain to any gene locus by fusing it to an appropriately specific DNA-binding domain (DBD). Thus, one can take advantage of the entire repertoire of characterized transcription-regulatory domains to achieve precisely the level and type of regulation desired for the gene of interest.

2. Engineering zinc finger protein-based transcription factors

The practical implementation of such an approach requires the availability of methods for selecting or designing DBDs with novel, pre-determined DNA-sequence specificities. Such methods could, in principle, involve any of the large number of naturally occurring DNA-binding motifs, or even artificial DNA-binding modules with novel protein folds [23]. Most such methods described to date, however, have involved the Cys_2–His_2 zinc finger [22,24]. Several features of the Cys_2–His_2 zinc finger make it a uniquely attractive scaffold for the purpose of designing DBDs with desired specificity. Foremost among these is its exceptional adaptability. Site selection studies of natural zinc finger proteins (ZFPs) have demonstrated that this motif may be adapted to exhibit a diverse range of DNA sequence specificities (for example, see [25–32]). Furthermore, unlike many other transcription factors, Cys_2–His_2 zinc finger proteins can function as monomers and their binding sites are therefore not restricted to palindromic sequences. Taken together, these two attributes suggest that there may be no inherent limitations to our ability to target ZFPs to new chromosomal locations. A second attractive feature of the Cys_2–His_2 zinc finger is its mode of base recognition, as revealed in structural studies of ZFP–DNA complexes [21,33–39]. In docking with DNA targets, ZFPs coded in mammalian genomes use multiple, tandem fingers to interact with a series of adjacent subsites in the major groove which are each typically three of four base pairs in length. This arrangement, coupled with observations of few direct contacts between adjacent fingers, provides a degree of functional modularity in the recognition by each finger of its cognate subsite target. This simplifies the problem of designing or selecting proteins with new DNA sequence specificities, since it permits one to optimize base recognition one finger (and one subsite) at a time [40,41] (Fig. 1).

The modular nature of zinc finger–DNA recognition has enabled the successful application of ‘mix and match’ approaches to DNA-binding protein design, in which fingers with known subsite preferences are linked together to yield multifinger proteins with high affinity for desired target sequences [42–49]. These approaches have benefited from the availability of a large panel of fingers with known sequence preferences, some of which have been derived from well-characterized natural proteins, but most of which have been the product of selection or design efforts aimed at modifying zinc finger specificity [40,41,43–45,50–60].

‘Mix and match’ approaches have employed a variety of linkages to connect fingers to yield multifinger proteins of desired composite specificity and affinity including the canonical TGEKP interfinger linker (or highly related sequences) [42,44–49,59,61] and a variety of longer more flexible linkers [40,62–64], as well as dimerization

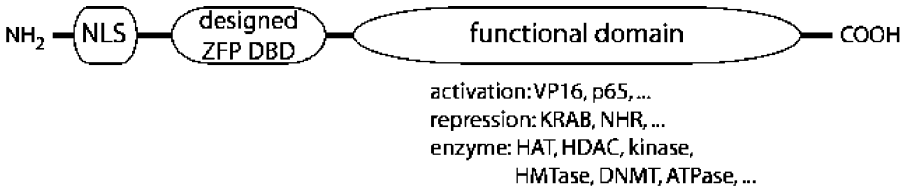

Fig. 1. Schematic representation of one possible architecture of a designed transcription factor, in which a nuclear localization signal (NLS) is followed by a DNA-binding domain (DBD)-based on the zinc finger protein, a functional domain (a small number of the wide range of possibilities are indicated), and a tag to facilitate purification and/or immunological detection.

domains [65–67]. These studies have been successful at functionally linking from three to six fingers to obtain recognition of targets ranging in length from 9 to 18 bp.

In specific cases, the simple obstruction of a particular sequence within a target gene promoter may have the desired functional effect. For example, Bartsevich and Juliano report that repression of the *MDR1* promoter can be achieved with a designed ZFP that occludes an Sp1 site [40]. One imagines that gene activation could also, in principle, be achieved by occluding the binding site for a repressor. The next section reviews published examples of regulating endogenous mammalian genes using fusions of ZFPs to a variety of functional domains (Fig. 1).

3. Regulation of endogenous mammalian genes with designed ZFP transcription factors

It is important to distinguish regulation of transcription by designed regulators of endogenous copies of chromosomal genes (for example, *erbB-2* [42] or *EPO* [48]) from control of other kinds of templates—DNA transiently transfected or otherwise introduced into cells, templates of *in vitro* transcription reaction, etc. It has been known for a considerable amount of time [68] that the chromosomal environment is markedly different from any other milieu for transcription factor function [69]. A growing number of published reports describe use of designed ZFP transcription factors to regulate endogenous genes.

Bartsevich and Juliano have repressed transcription of the multidrug resistance gene (*MDR1*) in K562 cells by targeting its promoter with a five finger protein fused to two KRAB-A repressor domains [40]. In two related studies, Beerli *et al.* and Dreier *et al.* have demonstrated regulation of the *erbB-2* and *erbB-3* genes, achieving either activation or repression dependent on the type of functional domain tethered to the six-finger proteins used in the study [42,59]. In another study, Zhang *et al.*, successfully activated the erythropoietin (*EPO*) gene in HEK 293 cells [48], despite the fact that the erythropoietin locus is silent in these cells and is refractory to stimuli such as exposure to hypoxia which induce expression of this gene in other cell types [48]. Interestingly, designed activators directed against a stretch of linker DNA between two positioned nucleosomes were most effective in upregulating the *EPO* gene, indicating that, as expected from several other studies [70,71], chromatin makes an important contribution to regulating *in vivo* binding. Liu *et al.*, recently demonstrated activation of the vascular endothelial growth factor A gene (*VEGF-A*) using a panel of three-finger proteins targeted to sites throughout the promoter region of this gene [49]. In this study, proportional upregulation of all major VEGF-A splice variants was observed, an important concern given the role of isoform balance in the proper function of this gene [72,73].

The utility of designed transcription factor gene control for dissection of regulatory pathways and also the therapeutic promise of such regulation is well-illustrated by a recent study on the role of a nuclear hormone receptor, PPARγ, in adipogenesis [74]. A member of a large and extensively studied family of ligand-regulated transcription factors, PPARγ makes a major, albeit insufficiently understood, contribution to the production of fat tissue [75]—it is also a major target of therapeutic agents in the treatment of type II diabetes. The protein exists in two major isoforms produced off the same DNA locus by transcription initiation from distinct promoters: the γ2 isoform is found exclusively in adipocytes, while PPARγ1 has a broader expression pattern. A functional distinction between the two has been very hard to make, because the isoforms are identical except for a short stretch of uncertain function at the extreme NH_2-terminus. Ren *et al.*, describe the use of a six-finger ZFP designed against a stretch of remodeled chromatin (a ‘‘DNase I hypersensitive site’’) in the *PPARγ2* promoter to perform ‘‘mutation-free reverse genetics’’: they used a fusion of this ZFP to the KRAB repression domain to fully ablate induction of both PPARγ1 and γ2 by adipogenic signals—the action of the ZFP prevented the differentiation of pre-adipocytes [74]. Importantly, this differentiation block could be rescued by the reintroduction of PPARγ2 but not PPARγ1, indicating that the former plays a unique and critical role in adipogenesis [74].

In model organisms with robust reverse-genetic tools—for example, budding yeast and *C. elegans*—directed lesions or alterations in transcriptional control pathways have long been established as one of the most powerful techniques available for experimental analysis of genome function [76,77]. The published studies reviewed here on the use of designed ZFP transcription factors to control endogenous mammalian genes demonstrate the feasibility of ‘‘gene regulation on demand’’ in higher eukaryotes.

Integration of their use with data from the extraordinary recent studies of genome-wide behavior patterns in normal development and in disease [78] opens an entirely new set of opportunities for genome analysis and control. The growing use of transcription-oriented therapies in clinical practice [11] further illuminate the potential utility of imposed and highly targeted gene regulation in therapeutic settings.

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