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Self-propagating artificial transcription factors to enhance upregulation of target genes

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ABSTRACT

Zinc-finger-based artificial transcription factors (ATFs) have been used to regulate expression of target genes both in vitro and in vivo. However, if we develop ATF expression further, target gene regulation by ATFs may be more effective. Here, we report a new transcriptional regulation system in which an ATF that is designed to upregulate a target gene also activates itself. To construct the system, we inserted tandem copies of the ATF-binding sites upstream of a promoter for ATF expression. Using the endogenous human VEGF-A gene, we demonstrated that the new expression system amplified ATF expression in a manner dependent on the number of copies of the ATF-binding site, and that the 'self-propagating ATF' upregulated VEGF-A gene expression more efficiently than a control promoter with no ATF-binding site.

Zinc-finger-based artificial transcription factors (ATFs) that in principle comprise a nuclear localization signal, a transcription regulatory domain, and an artificial zinc-finger protein (AZP) as a DNA binder have successfully regulated expression of endogenous target genes both in vitro and in vivo.[1–8] An effectively engineered AZP allows ATFs to both up- and downregulate expression of target genes by switching the transcriptional regulatory domain (i.e., using a transcriptional activation domain or a repressor domain). ATFs are also able to regulate each splice variant of a target gene proportionally[3,4] because ATFs regulate transcription of target genes. Proper isoform balance is crucial for in vivo function of a gene product (e.g., vascular endothelial growth factor A {VEGF-A};[9,10]).

We previously constructed ATFs for the human VEGF-A gene to treat ischemic diseases and cancer, and demonstrated that our ATFs effectively up- and downregulated endogenous VEGF-A gene expression under both normoxic and hypoxic conditions.[5,11] However, if we develop ATF expression further, target gene regulation by ATFs may be more effective because a higher concentration of ATF increases its binding to the target gene, leading to enhanced upregulation of the target gene. To enhance exogenous transgene expression, strong promoters and/or introns have generally been used. In the present study, we developed a novel transcriptional regulation system in which an ATF that was designed to upregulate a target gene also activated itself. To construct the system, we inserted tandem copies of the ATF-binding site (ABS) upstream of a promoter for expression of the ATF. We first examined whether the new expression system amplified ATF expression. We then examined whether the 'self-propagating ATF' enhanced VEGF-A expression using the endogenous human VEGF-A gene.

In this study, our main goal was to develop a self-propagating ATF expression system by inserting tandem copies of an ABS upstream of a promoter in an ATF expression plasmid (Fig. 1A). We reasoned that once the promoter expresses an ATF, the ATF will bind not only to an ABS located in a target gene, but also to tandemly-repeated ABSs in its own expression plasmid, leading to amplification of the ATF. The self-amplified ATF was expected to enhance endogenous VEGF-A expression further because the amounts of ATFs available for upregulation of a target gene were increased.

At the same time, we aimed to upregulate endogenous VEGF-A specifically in hypoxia for future application to the treatment of ischemic diseases. To this end, we used the ATF previously generated for VEGF-A upregulation[5,11] and a hypoxia-driven promoter for expression of the ATF, which was composed of two tandem copies of an hypoxia-response element (HRE) (5′-CCACAGTGCA TACGTGGGCTCCAACAGGTCCTCTT-3′)[12] and a minimal cytomegalovirus (CMV) promoter (−53 to +7 in the CMV promoter, where +1 is the transcription start site).[13] To construct the self-propagating ATF expression plasmids pABS6HRE-ATF and pABS12HRE-ATF (Fig. 1B), we inserted six and twelve copies of the 19-bp ABS of 5′-GGG GCT GGG GGC GGT GTC T-3′ (+516 to +534 in the human VEGF-A gene, where +1 is the transcription start site)[14] respectively, which is located in the human *VEGF-A* 5′-UTR, into a hypoxia-driven promoter. We used pHRE-ATF containing no ABS and pcDNA3.1 containing no coding region as controls.

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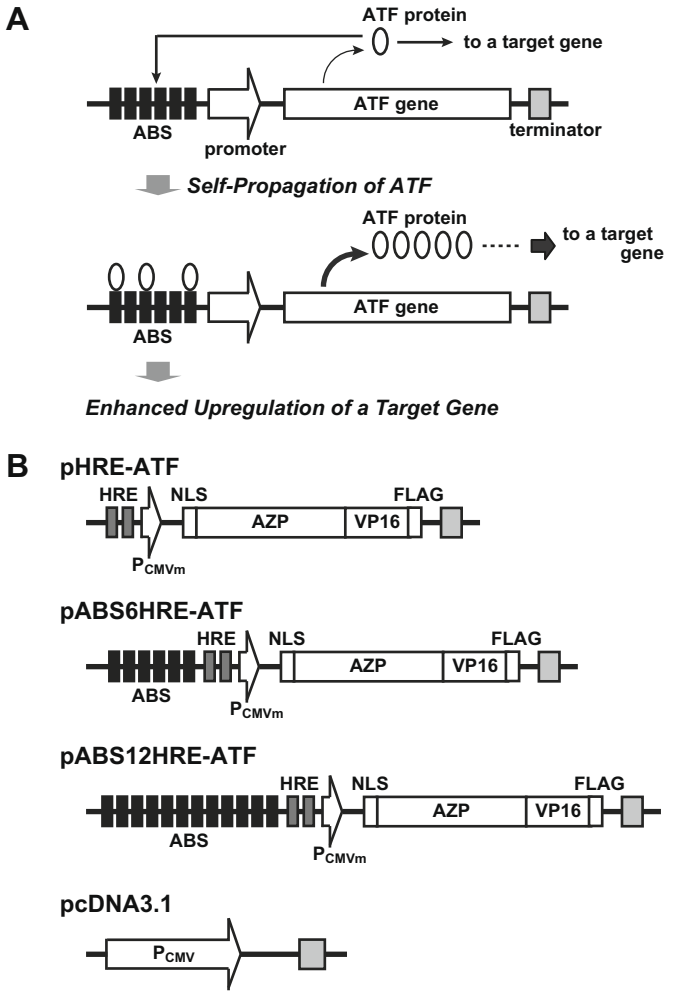

Figure 1. (A) Schematic description of the concept of self-propagation of ATF. The ATF binds not only to the ATF-binding site (ABS) in its target gene, but also to the ABSs in the ATF expression plasmid, leading to self-propagation of ATF. The amplified ATF is expected to enhance upregulation of a target gene because a higher concentration of ATF favors ATF-binding to its target gene. (B) ATF expression plasmids used in this study. The black boxes, dark gray boxes, short open arrows, light gray boxes, and open arrow indicate an ABS, a hypoxia-response element (HRE), minimal CMV promoter, BGH polyadenylation signal, and CMV promoter, respectively. The open boxes indicate open reading frames of a nuclear localization signal (NLS), an AZP, a VP-16 activation domain, and a FLAG epitope tag, but do not reflect actual sizes.

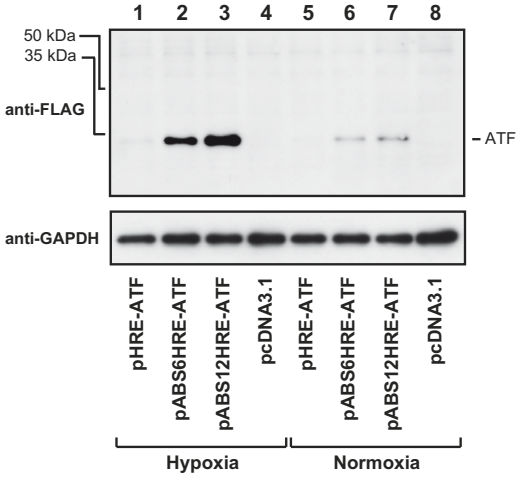

Figure 2. ATF expression amplified by self-propagating ATF expression systems. An ATF expression plasmid (or pcDNA3.1 as a control) (0.45 μg) and pCMV-β-galactosidase plasmid (0.05 μg) were cotransfected into 1×10^5 human embryonic kidney 293 cells by electroporation. After incubation at 37 °C for 24 h in hypoxia (1% O_2) or in normoxia, the culture medium and transfected cells were collected separately (the medium was used for analysis of VEGF-A protein production; see the legend of Fig. 3). The cell lysates were used for immunoblotting analysis. The molecular weight of the ATF is 32.2 kDa. The plasmid used for each cotransfection with pCMV-β-galactosidase and incubation atmosphere are indicated below the figure.

We first examined whether the newly designed expression systems actually amplified ATF expression. As shown in Figure 2, lane 1 in hypoxia (1% O_2), a very faint band of ATF was detected in transfection with pHRE-ATF, which did not contain any ABS. In contrast, the ATF expression level from pABS6HRE-ATF that harbored six ABSs was much greater than that from pHRE-ATF (compare lane 2 with lane 1 in Fig. 2). Moreover, the ATF expression was amplified further in pABS12HRE-ATF harboring 12 ABSs, indicating that the ATF amplification depended on the number of ABS copies (compare lane 2 with lane 3 in Fig. 2). Under the conditions, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was equally detected in all proteins samples. On the other hand, in normoxia, faint bands of the ATF were also detected in transfection with pABS6HRE-ATF and pABS12HRE-ATF (lanes 6 and 7 in Fig. 2), suggesting that ATF leaked from pHRE-ATF in normoxia was also amplified.

Next, we examined whether the self-propagating ATF expression systems upregulated endogenous VEGF-A gene expression more effectively than a control containing no ABS. In hypoxia, transfection of the pABS6HRE-ATF resulted in an 8.8 ± 1.6-fold activation of the endogenous VEGF-A gene compared with that of cells transfected with pcDNA3.1 containing no coding region (Fig. 3A). In contrast, transfection of the pHRE-ATF, which did not contain any ABS, resulted in a 4.4 ± 0.6-fold activation of the endogenous VEGF-A gene compared with that of cells transfected with pcDNA3.1 (Fig. 3A). Therefore, VEGF-A production induced by pABS6HRE-ATF was 2.0-fold compared with that induced by the control pHRE-ATF (P<0.05). Furthermore, pABS12HRE-ATF increased VEGF-A production by 9.8 ± 1.5-fold compared with pcDNA3.1, indicating that VEGF-A upregulation by the self-propagating ATF depended on the number of copies of the ABS as well. On the other hand, in normoxia (Fig. 3B), there was no statistically significant difference between VEGF-A production by pABS6HRE-ATF or pABS12HRE-ATF and that by the control pHRE-ATF (P = 0.31 and P = 0.34, respectively) although faint bands of the ATF were detected in the immunoblotting analysis described above (Fig. 2, lanes 6 and 7).

In this study, we showed that our self-propagating ATF expression systems effectively amplified their own ATF expression (lanes 2 and 3 in Fig. 2). The ATF expression levels depended on the number of copies of the ABS used in the expression system. This result indicates that the ATF expressed by the systems recognized the ABS in living cells and thereby amplified its own ATF expression, finally leading to enhanced upregulation of *VEGF-A*.

Although the increase in the number of ABS copies from six to twelve resulted in enhancement of ATF production (compare lane 2 with lane 3 in Fig. 2), such enhancement was not observed in VEGF-A production (8.8- and 9.8-fold activation by pABS6HRE-ATF and pABS12HRE-ATF, respectively, compared with pcDNA3.1). This result may be attributed to the fact that there are only two copies of the ATF's target sequence in the human genome. The self-propagating ATF expression systems may be more effective for upregulation of endogenous or exogenous multigenes.

We also demonstrated that the self-propagating ATF expression systems upregulated endogenous VEGF-A expression specifically in hypoxia (Fig. 3A) in combination with the hypoxia-driven promoter. By using the HRE, the hypoxic signal was amplified through ATF expression. Cis elements responsive to other biological signals

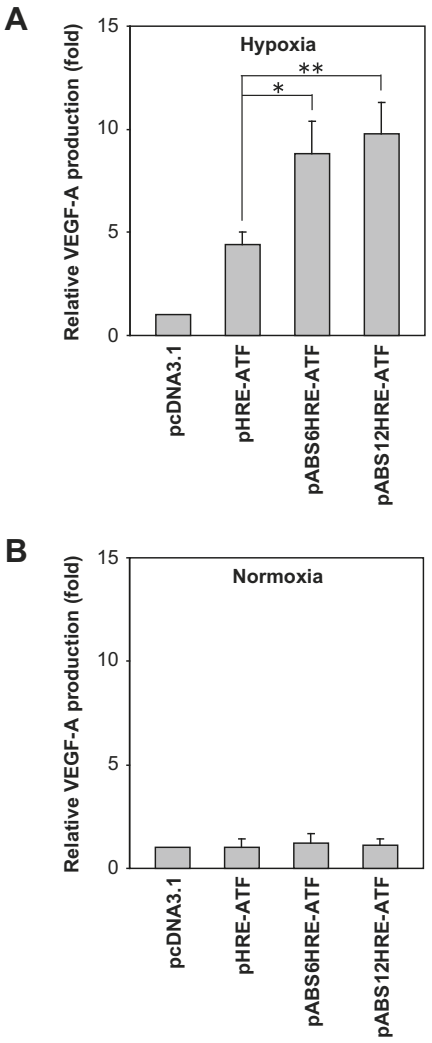

Figure 3. Enhanced upregulation of the endogenous human VEGF-A gene specifically in hypoxia by self-propagating ATF expression systems. (A) Enhancement of VEGF-A protein production by the self-amplified ATF expression systems in hypoxia. The VEGF-A concentration of the culture medium prepared for Figure 2 was quantified as described in Supplementary data. Each point represents the mean ± SD obtained from three independent experiments. The plasmid used for each transfection is indicated below the figure. Asterisk, P <0.05; double asterisk, P <0.01. (B) Effect of transfection with self-propagating ATF expression systems on VEGF-A protein production in normoxia. The VEGF-A concentration of the culture medium prepared for Figure 2 was quantified. Each point represents the mean ± SD obtained from three independent experiments. The plasmid used for each transfection is indicated below the figure. The difference between VEGF-A production by pABS6HRE-ATF or pABS12HRE-ATF and that by the control pcDNA3.1 was not statistically significant (P = 0.60 or 0.57, respectively).

including signal transduction may also be useful as elements of the inducible promoters for ATF amplification to enhance upregulation of genes depending on the biological signals.

On the other hand, the self-propagating ATF expression systems did not upregulate endogenous VEGF-A expression in normoxia (Fig. 3B) even though leaky ATF expression was detected by immunoblotting as shown in Figure 2 (lanes 6 and 7). At this stage, we do not understand the reason for this. However, one possible explanation might be that the target site in the *VEGF-A* 5′-UTR became more accessible to the ATF in hypoxia than in normoxia because histone demethylases JMJD1A and JMJD2B induced by the hypoxia-inducible factor HIF-1α in hypoxia are known to change chromatin structures.[15,16]

Abundant ATFs produced by self-propagation may be useful for increasing numbers of cells in which a target gene is regulated by ATF if we are able to transport ATFs intercellularly. Fusion of an ATF to an intercellularly transporting peptide/protein such as VP22[17–19] enables secretion of ATFs from cells transfected with the self-propagating ATF expression plasmid and then penetration into (untransfected) surrounding cells, thereby leading to a substantial increase in the total number of transfected cells. Such an intercellular ATF-transporting system may be especially useful for cells with low transfection efficiency such as primary cells.

In conclusion, we constructed self-propagating ATF expression systems, and demonstrated amplification of ATF expression by the new expression systems and more effective upregulation of VEGF-A expression by the self-propagating ATF. At the same time, we succeeded in hypoxia-specific upregulation of human *VEGF-A* by combining them with a hypoxia-driven promoter. We hope that our self-propagating ATF expression systems will be useful to refine technologies for gene regulation by itself or in combination with biological signals or other technologies.

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Supplementary data

Supplementary data (materials and methods) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.002.

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