Solid-Phase Synthesis

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An Integrated Chip for the High-Throughput Synthesis of Transcription Activator-like Effectors**

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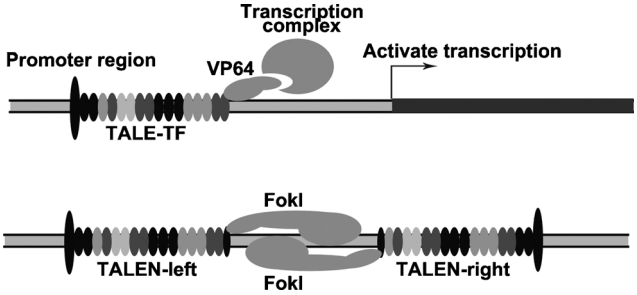
Recent developments using transcription activator-like effectors (TALEs) provide an alternative approach to the design and synthesis of sequence-specific nucleases.[1] TALEs, originally discovered in the plant pathogen *Xanthomonas sp.*, comprised of a variable number of tandem monomers (often 16 or more), each having 33–35 amino acids that specifically recognize one target nucleotide base.[2] The sequence of the monomers is highly conserved, but they differ primarily at amino acids 12 and 13.[3] This region, known as the repeat variable diresidue (RVD), is thought to determine the nucleotide-binding specificity of each TALE monomer (NI = A, HD = C, NG = T, NN = G or A).

The well-delineated specificity between the RVD and its target base makes TALEs a powerful DNA-targeting tool with various applications. For example, fusion of the FokI nuclease domains to the C-termini of two paired synthetic TAL effectors (giving TALE nucleases, TALENs) allows efficient DNA sequence-specific endonuclease activity in a variety of cells and organisms, including human stem cells, zebrafish, and rats (Scheme 1).[4] Once the paired TALENs bind to the target sites, the FokI catalytic domains dimerize between the paired binding sites and cleave the DNA, introducing double-strand breaks (DSBs). DSBs are generally repaired by the nonhomologous end-joining (NHEJ) pathway, which results in small deletions or insertions and functional gene knock-out. Alternatively, TALE-Fok1-mediated DSBs can also stimulate homologous recombination, enabling site-specific insertion of an exogenous DNA sequence.

TALE transcription factors (TALE-TFs) are constructed by fusion of the TALE DNA-binding domain with the VP64 transcription activation domain (Scheme 1); these factors bind a specific site in the promoter region of a gene upstream of the transcription start site and recruit the transcription complex to initiate gene transcription.



Scheme 1. Two applications for TALEs. (Top) TALEs can be used to generate customized transcription factors (TALE-TFs) and modulate the transcription of endogenous genes. The TALE DNA-binding domain is fused to the synthetic VP64 transcriptional activator, which recruits RNA polymerase and other factors required to initiate transcription. (Bottom) TALE nucleases (TALENs) can be used to generate site-specific DNA double-strand breaks and facilitate genome editing through nonhomologous end joining or homologous recombination. Each TALE DNA-binding domain is fused to the catalytic domain of the FokI endonuclease; upon FokI dimerization, the endonuclease cuts the DNA between the left and right TALEN binding sites.

Despite these applications, the capability to fully utilize TALEN or TALE-TF remains limited because of the difficulty in constructing customized TALEs with specific arrangements of tandem repeat monomers. A few strategies for constructing TAL effector repeats have been reported, such as "hierarchical-ligation assembly", "unit assembly", "modular assembly", "golden-gate clone", and others.[5] However, these methods require labor-intensive operations, including gel purification of products and selection of positive bacterial clones after each step of ligation.

By taking advantage of solid-phase gene synthesis, we report herein a novel strategy of magnetic bead-based TALE assembly, which allowed the synthesis of over one hundred TALEs, comprised of 16 or 20 repeat units, in three days. We used a chip containing two components: a microwell array and magnetic microbeads coated with streptavidin (Figure 1 A). Utilizing this chip allowed the simplification of large-scale TALE production to three steps: monomer ligation, enzymatic digestion, and purification (Figure 1 B).

Briefly, a biotinylated DNA double-strand adaptor with an SpeI restriction site on the one end was immobilized on streptavidin-coated magnetic beads. The first repeat unit was digested with SpeI and subsequently ligated to the adaptor using T4 DNA ligase on the magnetic beads. After rinsing with buffer three times, the ligated products (on the beads)

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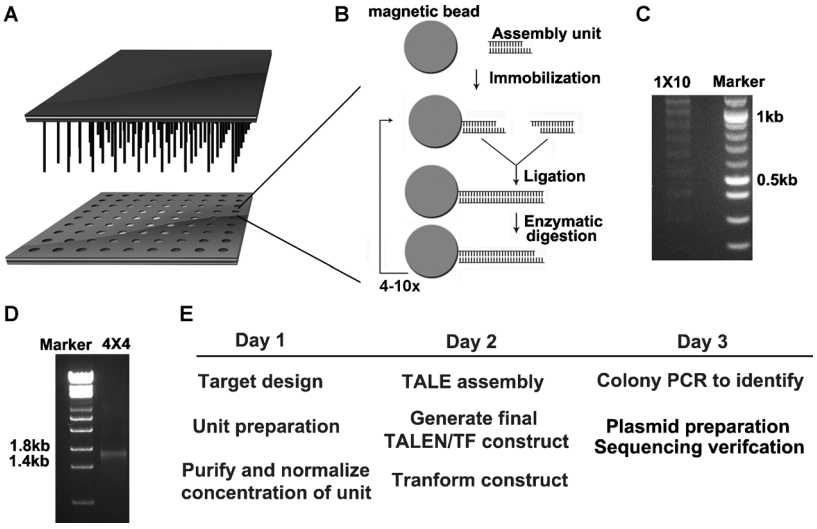

Figure 1. Large-scale synthesis of TALEs. A) The diagram shows the set-up for synthesis of TALEs using a robotic system. B) An outline of the synthesis steps. C) Image of an agarose gel showing the ligation products of ten consecutive steps of monomer-unit assembly. D) Image of an agarose gel showing the ligation products of four consecutive steps of assembly with a four-monomer unit. E) Schedule for the synthesis of TALEN/TALE-TF.

were then digested with NheI to regenerate an overhang for the next repeat unit to bind. The next repeat unit was ligated to the beads following the same protocol and the steps repeated. Since the ligation products were immobilized on beads, the digestion solution or ligation solution could easily be removed, leaving a pure product. Unlike traditional strategies of TALE assembly, in which a number of time-consuming and labor-intensive operations, such as gel-based purification and colony selection are needed, the solid-phase synthesis approach demonstrated herein allowed quick and simple purification of the ligation product.

To assess the efficiency of synthesis using the bead strategy, several different repeat units, including monomers, dimers, trimers, and tetramers, were used in the synthesis process. As shown in Figure 1C, the synthesis products appeared as a ladder, indicating a series of bands with different sizes. According to a quantitative analysis using Image J software, no less than 20% decamer product was obtained after ten rounds of ligation and digestion of monomer repeat units. On the other hand, more than 90% 16-monomer product could be obtained after four cycles with tetramer repeat units (Figure 1D). Using this strategy, the synthesis and sequencing of a 16-monomer product would take less than three days (Figure 1E).

Next, to demonstrate the sequence-specific binding activity of the synthesized TALE, we designed two assays, a TALEN assay employing TALE-FokI and a TALE-TF assay employing TALE-VP64. First, two pairs of TALENs were constructed and used to introduce double-strand breaks (DSBs) in either an exogenous gene, the luciferase single-strand annealing (SSA) reporter gene, or an endogenous gene, the monoamine oxidase A gene (MAOA).

We used the luciferase SSA reporter gene to demonstrate the repair pathway through homologous recombination after DSBs, which is comprised of two truncated luciferase fragments, each containing an 870 bp homologous arm, and separated by a stop codon and the TALEN target site (Figure 2A). Upon targeted DNA cleavage, luciferase activity could be recovered through SSA and homologous recombination repair. Compared with an empty vector in the control experiment (NC), luciferase activity was increased fivefold upon co-transfection of the two paired TALE-FokIs and the luciferase SSA reporter (Figure 2B).

To demonstrate the repair of DSBs induced by TALENs through nonhomologous end joining, we next constructed a pair of TALE-Fok1 nuclease expression plasmids targeting MAOA and transfected them into 293T cells. To detect cleavage efficiency, we first used PCR to amplify a 450 bp DNA fragment spanning the TALEN target site. A single BclI restriction site is located between the TALEN binding sites; in the wild-type DNA, complete BclI digestion of the PCR product produced two fragments (255 bp and 195 bp), as shown in control experiments (NC, Figure 2C). In contrast, successful TALEN-mediated cleavage and subsequent NHEJ DNA repair resulted in removal of the BclI site, thus generating a PCR product of approximately 450 bp. In fact, more than 50% intact DNA fragment was detected in the samples transfected with TALENs. Together, these results confirm that our synthetic TALENs could induce DNA DSBs and activate DNA repair pathways, through either homologous recombination or nonhomologous end joining in vivo.

For the TALE-TF assays, we constructed an expression vector for TALE-VP64 (pCS2-TALE-VP64), which was designed to bind a specific site upstream of the GFP open-reading frame in a sensor plasmid, referred to as miniCMV-Target (Figure 3A and Table 1). A stably transfected cell line containing the miniCMV-Target was established by lentivirus

Table 1: TALE target site sequences and the resulting RVDs (amino acid:nucleotide binding NI = A, HD = C, NG = T, NN = G).

TALE name	Target sequence	RVD
MAOA-TALE-L	CCTGGTTAAGATTACT	HD HD NG NN NN NG NG NI NI NN NI NG NG NI HD NG
MAOA-TALE-R	GGAGCATCTTCATCTT	NN NN NI NN HD NI NG HD NG NG HD NI NG HD NG NG
Target-TALE	CTTACACTTTCACGAAT	HD NG NG NI HD NI HD NG NG NG HD NI HD NN NI NI NG
NC-TALE	ACCTTACTATTCAAACT	NI HD HD NG NG NI HD NG NI NG NG HD NI NN NI HD NG
ROCK1-TALE	CTCCTCGTCAGAAGTCT	HD NG HD HD NG HD NN NG HD NI NN NI NI NN NG HD NG

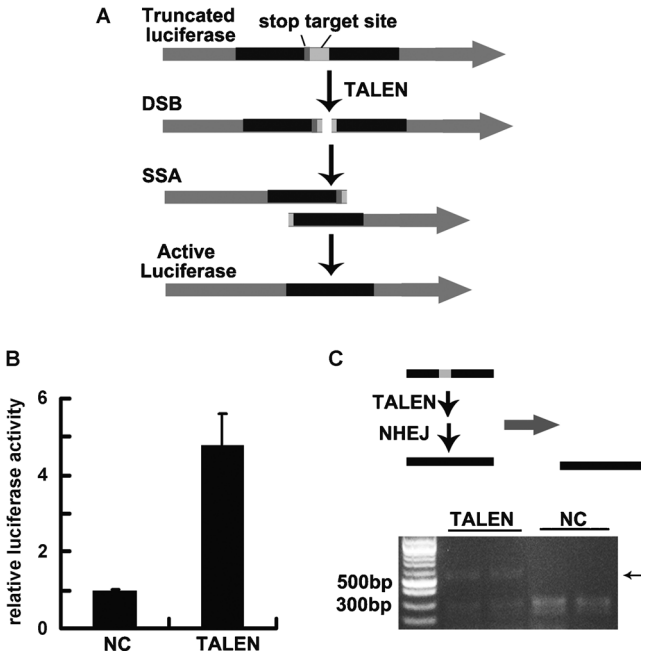

Figure 2. Detection of TALENs activity using a luciferase SSA recombination assay. A) A diagram of the luciferase SSA recombination assay is shown. A fragment containing a stop codon and a TALEN target site were flanked by two truncated firefly luciferase genes. Because these two truncated firefly luciferase coding regions each contain a 870 bp homologous arm (black), once TALENs bind and induce a DSB, DNA repair through SSA followed by homologous recombination between the two homologous arms occurs, thus producing active luciferase. B) SSA recombination assay showing luciferase activity before and after cleavage of the target site by TALENs. The luciferase activity increased fivefold when introducing TALEN relative to an empty vector (NC). Error bars indicate S.D., $n=3$. C) Successful TALEN-mediated cleavage of the TALEN target site in MAOA. In control experiments, the BclI digested PCR products consist of two fragments, approximately 255 bp and 195 bp. TALEN-mediated NHEJ resulted in the abolishment of a BclI site, thus the repaired fragment was resistant to BclI cleavage (400 bp, indicated by arrow).

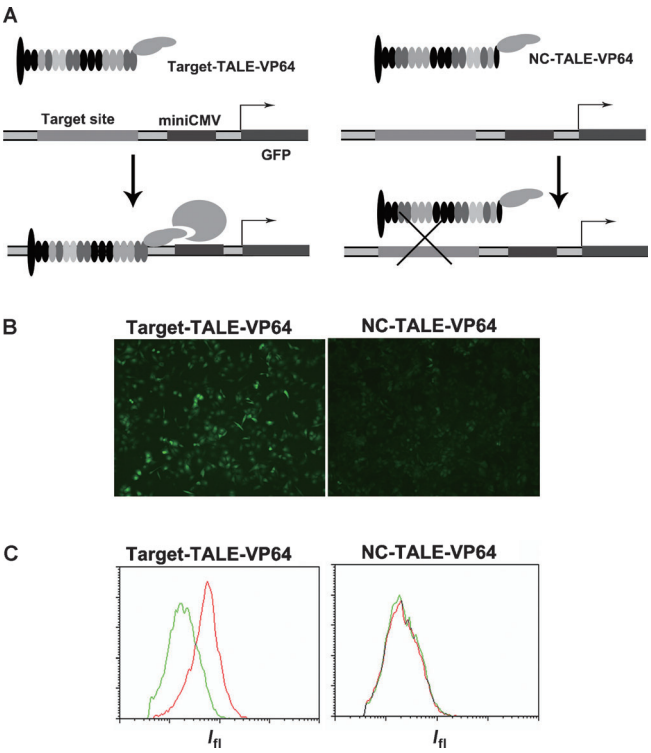

Figure 3. TALE-TF mediated transcriptional regulation in HeLa cells. A) Diagram of activation of the miniCMV promotor following transfection of Target-TALE-VP64 (or lack of activation with NC-TALE-VP64) into a stably transfected cell line. B) Fluorescent microscopic images were recorded after 48 h. C) Cells were analyzed using FACS analysis. Green = 0 h, red = 48 h. I_{fl} = fluorescence intensity measured on a logarithmic scale from 0 to 4.

infection. We transfected either Target-TALE-VP64 or a control sequence NC-TALE-VP64 into the miniCMV-Target cell line. After 48 h, the cells transfected with the Target-TALE-VP4 plasmids showed significantly enhanced GFP fluorescence in comparison with the control NC-TALE-VP64 (Figure 3B). Additionally, we quantitated these results by FACS analysis before and after transfection with pCS-TALE-VP64. Results showed that the median fluorescence intensity increased about 2.6-fold in the presence of Target-TALE-VP64 (Figure 3C).

Finally, we examined whether a new TALE-TF could specifically amplify an endogenous gene, thus showing potential for use in a screen for gain-of-function mutations. A previous study showed that up-regulation of Rho/ROCK signaling in tumors is linked to increased invasion and metastatic potential.[6] We constructed an expression vector for TALE-VP, which binds a specific site on the promoter of the ROCK1 gene and amplifies its expression. Consistent with the previous report, transfection of TALE-VP-ROCK1 resulted in a twofold increase in the migration of HeLa cells through a membrane; ROCK1 siRNA clearly inhibited the cell migration (Figure 4).

In summary, here we have presented a novel and robust approach for large-scale synthesis of TALE. This approach

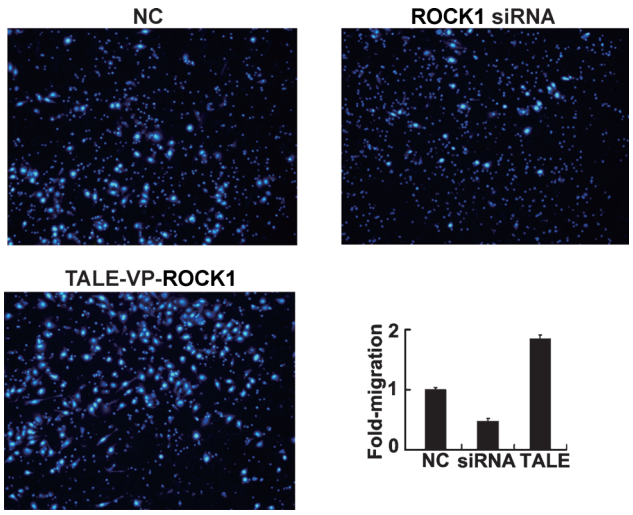

Figure 4. Fluorescent microscopic images of DAPI-stained HeLa cells following transfection with a control plasmid (NC), ROCK1 siRNA, or TALE-VP-ROCK1 and migration through a membrane. Bottom right: quantitation of the cell images shown here.

has certain unique features. Compared to most traditional approaches that necessitate labor-intensive gel purification or cloning steps; through the implementation of solid-phase synthesis, our approach significantly simplifies the purification of ligation products. Furthermore, we demonstrated two applications, genome editing and transcription modulation. Indeed, although TALE demonstrates potential for research or industrial applications, a number of concerns needed to be addressed. Our approach provides a powerful tool for synthesis of a large number of TALEs within a short period of time, thus providing a solid foundation for future studies and applications.

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