Inducing Stem Cell Myogenesis Using NanoScript

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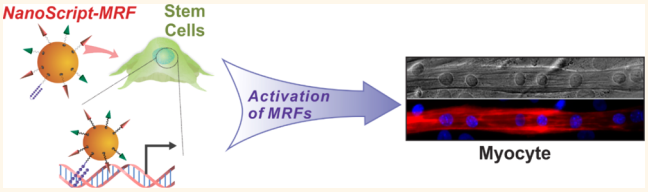
ABSTRACT Transcription factors (TFs) are multidomain proteins that play a critical role in orchestrating stem cell differentiation, but several limitations hinder the full potential of TF-based gene regulation. Here we report a unique strategy to emulate TFs and differentiate stem cells in a nonviral approach using an artificial, nanoparticle-based transcription factor called NanoScript. The NanoScript platform consists of a gold nanoparticle functionalized with small molecules that mimic the various domains of TFs. As a result, NanoScript mimics the function and structure of TF proteins. Specifically, NanoScript was designed to regulate muscle cell differentiation by targeting myogenic regulatory factors (MRFs), which play an important role in inducing myogenesis. This NanoScript-MRF is stable in physiological environments, localizes within the nucleus, induces differentiation of adipose-derived mesenchymal stem cells into mature muscle cells in 7 days, and is naturally excreted from induced muscle cells. As such, NanoScript represents a safe and powerful tool for applications requiring gene manipulation.



KEYWORDS: myogenesis · stem cell differentiation · nanoparticle-based genetic manipulation · transcription factors · nonviral delivery

Stem cell engineering and cellular reprogramming for regenerative medicine holds tremendous potential for treating many debilitating diseases and degenerative disorders.[1] As such, significant effort has been invested into developing methods to effectively control the differentiation of stem cells into specific lineages.[2] To this end, a subset of proteins called transcription factors (TFs) have been identified as the critical elements that orchestrate stem cell differentiation and cellular reprogramming.[3] The multidomain structure of TFs is composed of three essential domains: (i) the DNA-binding domain (DBD) that binds to specific DNA sequences, (ii) an activation domain (AD) that recruits RNA polymerase II and other proteins to initiate transcriptional activity, and (iii) a nuclear localization domain to enable entry into the nucleus. Through the synergistic activity of these domains, TFs interact with DNA to regulate gene expression, which, in turn, regulates fundamental cellular behaviors and can also override cellular identity to reprogram or differentiate cells into specific lineages.[4] For example, the process of generating muscle cells, known as myogenesis, is governed by a group of four TFs called myogenic regulatory factors (MRFs), which include *MyoD*, *Myogenin*, *Myf5*, and *Mrf4*, and have been demonstrated to play a critical role in generating muscle cells from both somatic and stem cells.[5−7] As such, the modulation of MRFs holds tremendous potential to treat incurable degenerative muscle disorders, such as muscular dystrophy.[8]

To date, most conventional methods for inducing stem cell differentiation or cellular reprogramming have been developed to either (i) deliver gene-specific TF proteins into cells (using nanocarriers, electroporation, and liposomes)[9−11] or (ii) stimulate the production of gene-specific TFs within the cells (using viral vectors and small molecules).[12−14] However, these methods are limited due to inherent drawbacks such as low delivery efficiency, lack of cell/nuclear-targeting capabilities, minimal gene expression, random genomic integration, and vulnerability to intracellular degradation.[15] Moreover, direct delivery of TF proteins into cells faces limitations, including intracellular degradation and low delivery efficiency, which leads to limited gene expression.[16] Hence, there is an urgent need to develop an alternative approach that

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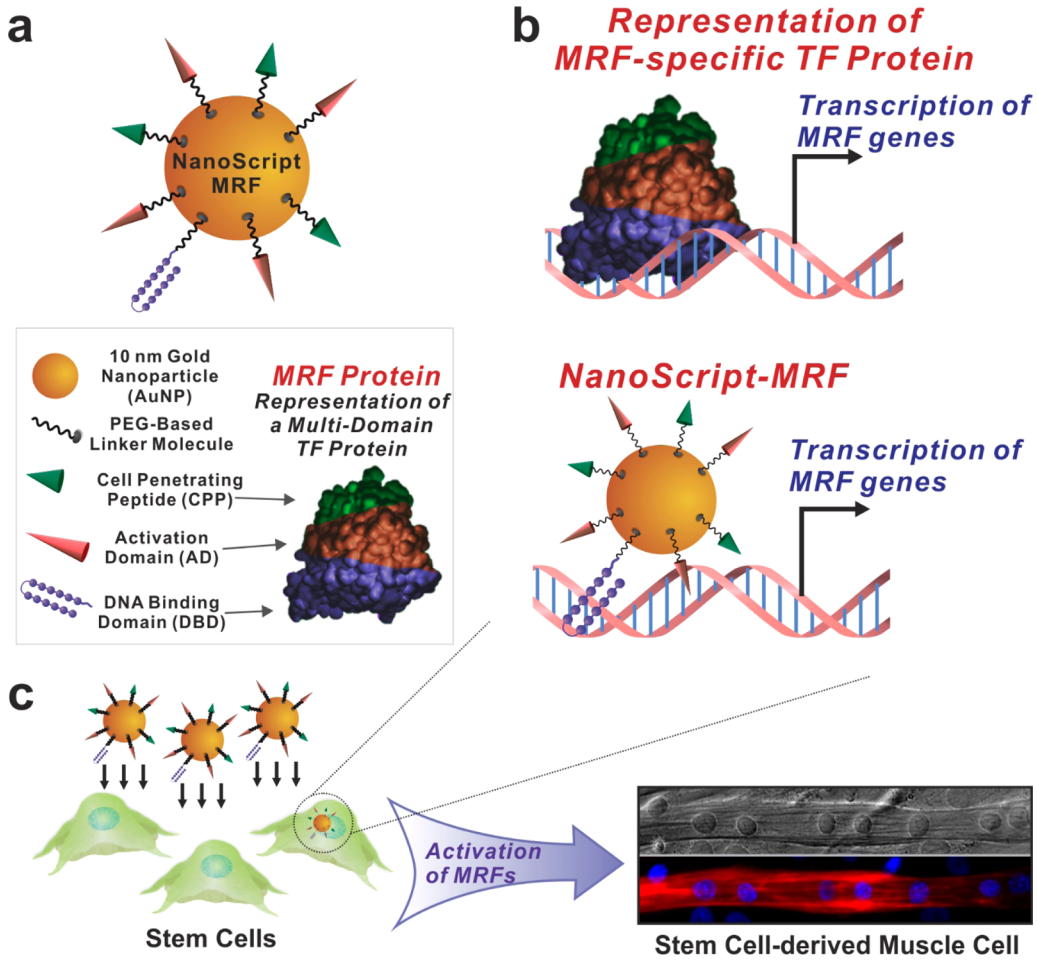
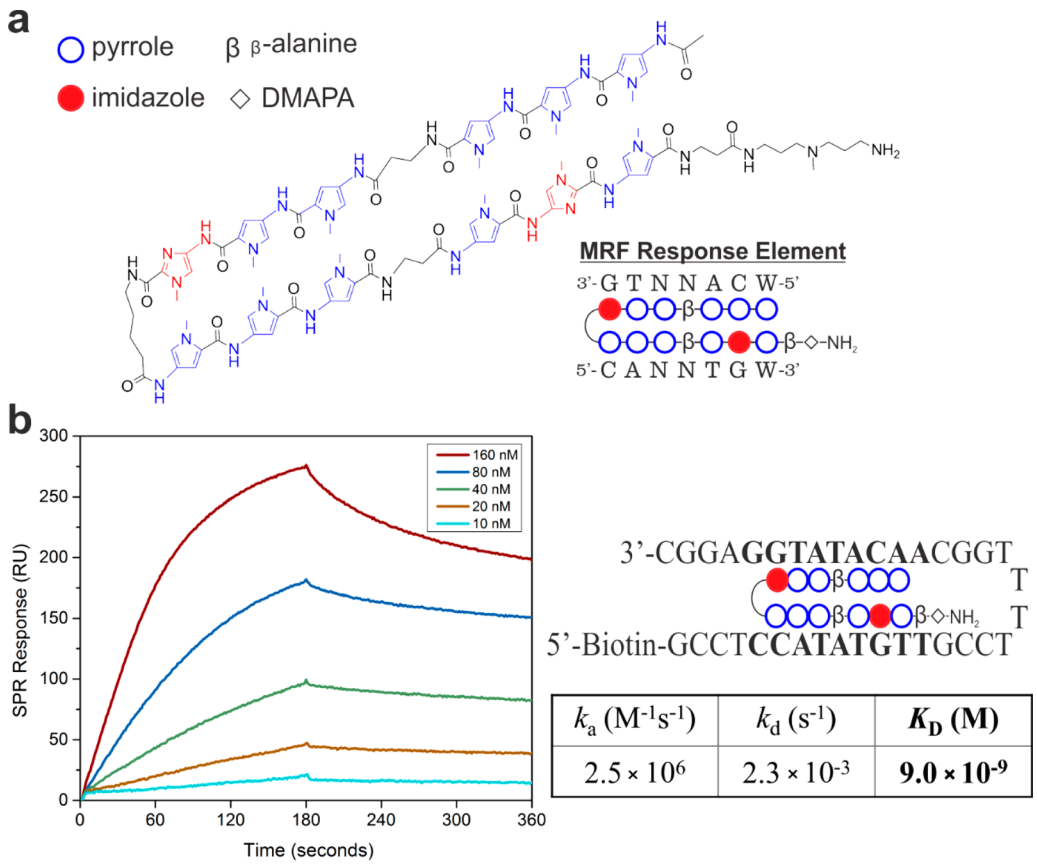


Figure 1. Schematic representation of NanoScript-MRF emulating transcription factor proteins. (a) Individual domains of TF proteins (such as the CPP, AD, and DBD) are mimicked through small molecules and peptide counterparts. By assembling these specific biomolecules on AuNPs, the NanoScript platform is fabricated. (b) Myogenic regulatory factors, which regulate muscle cell differentiation, are TFs that interact with DNA to regulate gene expression for muscle-specific genes. The NanoScript-MRF platform, which mimics MRFs, can perform the same function of expressing muscle-specific genes. (c) Transfection of NanoScript-MRF into adipose-derived mesenchymal stem cells induces activation of MRFs, which guides their differentiation into muscle cells.

not only overcomes the limitation of conventional methods but also is capable of regulating gene expression and inducing stem cell differentiation in an effective and nonintegrative manner.[16]

To this end, we recently demonstrated that by assembling functional molecules onto gold nanoparticles (AuNPs), an artificial nanoparticle-based transcription factor, called *NanoScript*, could be developed for effective gene regulation.[17] A remarkable feature of this NanoScript platform is its ability to behave and function just like natural TFs, as we recently demonstrated that NanoScript can initiate transcription of a reporter plasmid and overexpress targeted endogenous genes in a nonviral manner. While this proof-of-concept demonstration has established NanoScript as being capable of regulating gene expression, application of NanoScript for inducing stem cell differentiation remains unexplored. Due to the tunable properties of NanoScript, we hypothesize that this platform can be modified and utilized to mimic differentiation-specific TFs, such as MRFs, to induce stem cell differentiation.

In this report, we demonstrate that NanoScript can be designed to replicate natural TFs that are specific for myogenic regulatory factors (MRFs) (Figure 1). This NanoScript-MRF efficiently penetrates the plasma membrane and, with the help of a nuclear-targeting peptide, can localize within the nucleus while remaining intact. Importantly, NanoScript-MRF can target and activate the four critical genes that regulate myogenesis, which include *MyoD*, *Myogenin*, *Myf5*, and *Mrf*, in a nonintegrative approach to successfully differentiate adipose-derived mesenchymal stem cells (ADMSCs) into muscle cells. The gene expression levels induced by NanoScript-MRF are comparable to and even exceed those of conventional TF–protein-based delivery methods. Finally, we show that NanoScript-MRF is naturally cleared from the induced muscle cells. In this way, we have developed a modified NanoScript platform to effectively induce stem cell differentiation.



k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
2.5×10^6	2.3×10^{-3}	$\mathbf{9.0 \times 10^{-9}}$

Figure 2. MRF-DBD polyamide has high binding affinity for the target sequence. (a) MRF-DBD, with imidazole (red) and pyrrole (blue) motifs arranged in a specific sequence to complement the MRF response element (*N* = any base pair). (b) Sensorgrams show the interaction of varying polyamide MRF-DBD concentrations with the complementary hairpin DNA. The equilibrium constant (K_D), which is indicative of the binding affinity, was determined by the ratio of the dissociation constant (k_d) to the association constant (k_a).

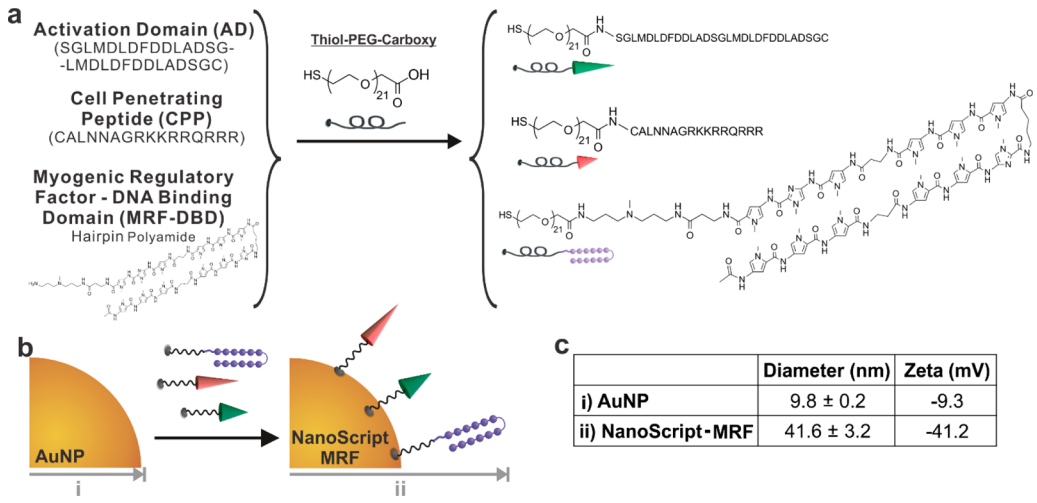
Moreover, NanoScript has tunable components that can easily be resequenced to target almost any gene of interest, and hence, it can mimic other transcription factors of interest.

RESULTS AND DISCUSSION

Design and Characterization of NanoScript-MRF. The NanoScript platform, designed to replicate the multidomain structure of natural TFs, comprises several key components: (1) a hairpin polyamide small molecule that mimics the DBD, (2) a peptide that mimics the AD, (3) a cell-penetrating peptide (CPP) to facilitate entry into the cell and nucleus, (4) polyethylene glycol (PEG)-based molecules to increase the stability of NanoScript in physiological conditions, and (5) a gold nanoparticle that acts as the linker domain to tether these components.

The first small molecule that we synthesized was a hairpin polyamide DBD, which comprises pyrrole (Py) and imidazole (Im) motifs that complement the A–T and G–C DNA base pairs, respectively, and can be designed to target specific gene sequences.[18,19] Natural MRFs activate muscle-specific transcription by binding to the DNA consensus sequence that is specific in the promoter region of all four MRF genes (Supporting Information Figure S1).[20] Hence, using an established solid-phase synthesis procedure,[21] we synthesized a hairpin polyamide DBD specific for MRFs (termed MRF-DBD) with the sequence PyPyPy-β-PyPyIm-γ-PyPyPy-β-PyImPy-β-Dp (γ is γ-aminobutyric acid, β is β-alanine, and Dp is dimethylaminopropylamide) to complement the consensus promoter sequence (Figure 2a). After synthesis, we used surface plasmon resonance to confirm that the MRF-DBD had a strong binding affinity of 9.0×10^{-9} M to its target sequence (Figure 2b). Then, to represent the AD, we designed a peptide referred to as a transactivation peptide (TAP), which has been shown to act as a potent activator and inducer of transcriptional activity.[22,23] Finally, an established CPP was designed to facilitate nuclear localization of NanoScript.[24]

In order for the NanoScript platform to behave and function like natural TFs, it must be highly soluble in aqueous solutions, stable in physiological conditions, and cannot form aggregates. For this purpose, PEG-based molecules are used because they significantly increase solubility of conjugated biomolecules and stabilize nanoparticles in physiological conditions.[25] Furthermore, thiol-terminated PEG molecules enable functionalization of a mixed monolayer on AuNPs.[24,26] Hence, we conjugated all three biomolecules (MRF-DBD,



	Diameter (nm)	Zeta (mV)
i) AuNP	9.8 ± 0.2	-9.3
ii) NanoScript-MRF	41.6 ± 3.2	-41.2

Figure 3. Conjugation of molecules and construction of NanoScript-MRF. (a) Each biomolecule (AD, CPP, and MRF-DBD) was conjugated to the thiol-PEG-carboxy linker molecule. (b) Three PEG-conjugated biomolecules were assembled on AuNPs *via* thiol moieties to construct the NanoScript-MRF, and (c) characterization using a zetasizer confirmed the diameter and surface charge of NanoScript.

TAP, and CPP) to a thiol-terminated PEG molecule, and then the three PEG-conjugated biomolecules were mixed with 10 nm AuNPs to construct NanoScript-MRF (Figure 3a,b). Using a zetasizer, the hydrodynamic diameter of the final NanoScript-MRF was determined to be 41.6 nm and the surface charge to be −41.2 mV (Figure 3c). Using high-performance liquid chromatography, the amount of ligands per gold nanoparticle was found to be 1297 ± 102 (Supporting Information Figure S2), which is in correlation with previous reports.[27] Furthermore, the solubility of NanoScript-MRF in physiological environments was evaluated by incubation in cell culture media for 7 days. Specifically, we found that the NanoSript-MRF maintained its monodisperse properties as compared to day 0 (Figure S3), thus confirming that NanoScript-MRF is suitable for cell applications.

NanoScript-MRF Localizes in the Nucleus. All transcriptional activity occurs exclusively inside the nucleus; hence, an important criterion for NanoScript to regulate gene expression is efficient membrane permeation and nuclear localization. To this end, we carefully ensured that NanoScript has two distinct features. The first feature is a specially designed CPP on NanoScript to facilitate permeation of both the plasma and the nuclear membrane.[24,28] The second feature is that the hydrodynamic diameter is smaller than 44 nm, which is the size of the nuclear pore.[29] Taken together, this ensures that NanoScript can specifically target and enter the nucleus.

NanoScript's ability to permeate the plasma membrane was evaluated by incubating NanoScript-MRF with ADMSCs. In order to calculate the concentration of NanoScript to be incubated with the ADMSCs, we performed a dose-dependent cell viability assay that revealed that the highest concentration with the highest viability is 10 μg/mL, and hence, we used this concentration for all subsequent experiments (Supporting Information Figure S4). To monitor intracellular localization, NanoScript was modified with a fluorescent dye and incubated in ADMSCs. Fluorescence imaging confirmed that NanoScript was able to translocate within the nucleus, as evidenced by the overlap of the Alexa Fluor 568 dye (attached onto NanoScript) and 4′,6-diamidino-2-phenylindole (DAPI) (nucleus staining) (Supporting Information Figure S5). Overall, an analysis of the intracellular localization dynamics of NanoScript-MRF confirmed that the hydrodynamic size of NanoScript-MRF (diameter = ~40 nm) is small enough to enter and accumulate within the nucleus.

NanoScript Induces Myogenesis from Stem Cells. In order to establish the NanoScript platform for stem cell differentiation, we evaluated NanoScript's ability to genetically reprogram ADMSCs into muscle cells.[30] Even though NanoScript can be applied to almost any cell line, ADMSCs were chosen because they are an excellent model of multipotency, and depending on which genes are activated, ADMSCs can differentiate into multiple lineages including muscle, bone, fat, and even neuronal cells.[13,31,32] Hence, we predicted that by activating MRFs in ADMSCs, particularly *MyoD* because it has been established as the most prominent myogenesis gene,[7,33] using NanoScript-MRF, we can guide their differentiation to generate muscle cells. When we adopted a differentiation timeline from a previous report,[13] ADMSCs were treated with NanoScript-MRF by incubating them in the culture media,[24] and then a myogenic medium was introduced to support muscle growth (Figure 4a). After 7 days, expression of muscle-specific markers and phenotype changes was analyzed. As evidenced by the expression of *Myogenin*,[34] a ubiquitous myogenic marker, and *myosin*,[13] a distinct muscle marker, we observed

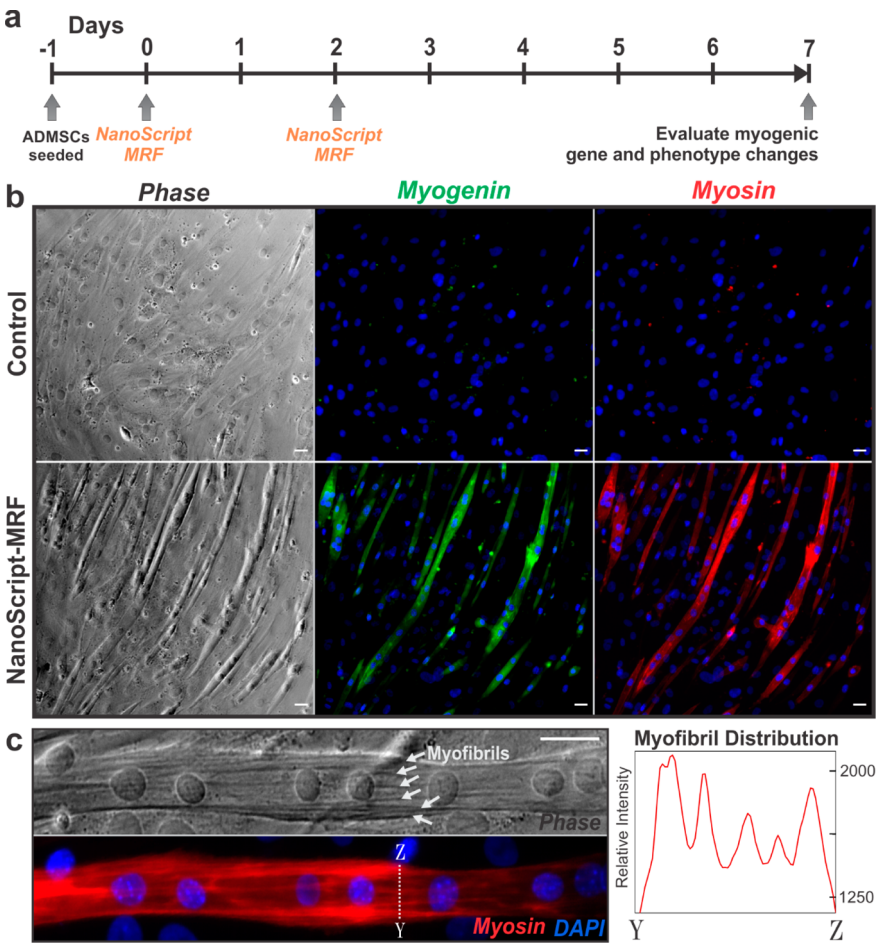


Figure 4. NanoScript-MRF generates muscle cells as evidenced by phenotypic and genotypic changes. (a) Differentiation timeline of ADMSCs being treated with NanoScript-MRF. (b) Phase and fluorescence images of ADMSCs that were fixed and stained on day 7. The untreated control ADMSCs (top row) and the NanoScript-MRF-treated ADMSCs (bottom row) were stained for muscle-specific markers such as myogenin (green, middle column) and myosin (red, right column) and the nucleus marker, DAPI (blue). (c) Magnified phase and fluorescence image of a multinucleated muscle cell shows the development of myofibrils (indicated with white arrows). A fluorescence intensity profile of the induced muscle cell shows the distribution of six distinct peaks for individual myofibrils (scale bar = 20 μm).

generation of myocytes, which are elongated, tubular muscle fiber cells, using the NanoScript-MRF platform (Figure 4b). This result was compared to the untreated control, which also included replacement with myogenic media and showed minimal expression of myogenic markers (Figure 4b). Additional control experiments evaluated the contribution of each domain on NanoScript. Because these control experiments, which included a NanoScript lacking an AD or MRF-DBD, were unable to significantly activate myogenic genes beyond basal levels, it signifies that the domains on NanoScript are important for initiating transcriptional activity (Supporting Information Figures S6 and S7). Furthermore, the induced myocytes displayed the unique phenotype of mature muscle fiber characteristics, as evidenced by the distribution of multiple nuclei and the formation of striations in the aligned muscle fiber. The formation of these striations, called myofibrils, was further observed in the fluorescence intensity profile, which showed six distinct peaks that represent individual myofibril filaments (Figure 4c).

This myogenic differentiation was further confirmed using qPCR to quantify the activation of muscle-specific genes. Two distinct intermediate myogenic genes, *desmin* and *enolase*,[13,35] along with all four MRFs were effectively activated in cells treated with NanoScript-MRF, as compared to the control (Figure 5a). Furthermore, the late-stage myocyte marker, *myosin heavy chain* (*MYH1*),[36] was up-regulated by about 28-fold (Figure 5a). This evidence strongly suggests that the gene expression potency induced by NanoScript-MRF is sufficient to trigger downstream myogenic genes involved in generating and supporting myocyte growth. As a result of activating myogenic genes, multipotency genes distinct for ADMSCs were suppressed and significantly down-regulated, thus indicating that differentiated cells no longer genetically resemble ADMSCs (Figure 5b). Moreover, cell viability assays indicated the viability of generated muscle cells to be 97.6 $\pm$ 1.1% (statistics from three independent experiments), as compared to the untreated control. Furthermore, we tested if NanoScript had any adverse

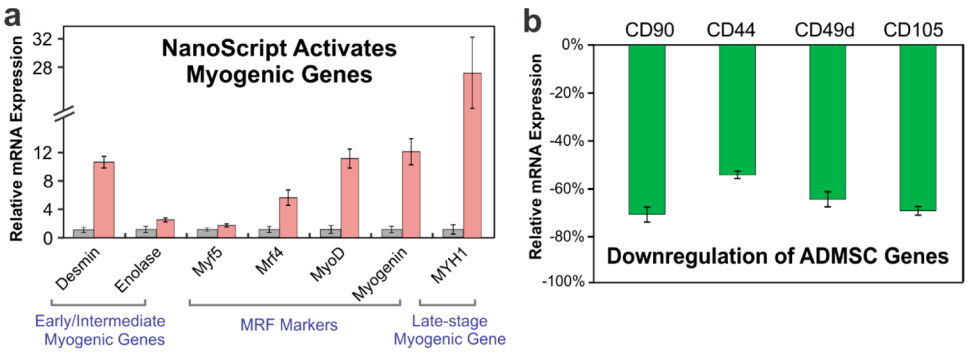


Figure 5. NanoScript-MRF initiates transcription and activation of myogenic genes. (a) After 7 days post-treatment with NanoScript-MRF, qPCR analysis reveals overexpression of targeted MRFs as well as significant expression of the late-stage myogenic gene *MYH1* by NanoScript-MRF (red) as compared to the untreated control (gray). (b) Multipotent genes of ADMSCs were down-regulated as compared to untreated control cells, thus indicating that stem properties of ADMSCs are repressed when myogenic genes are overexpressed. Error bars are from three independent trials, and mRNA expression is represented as a fold change compared to the control.

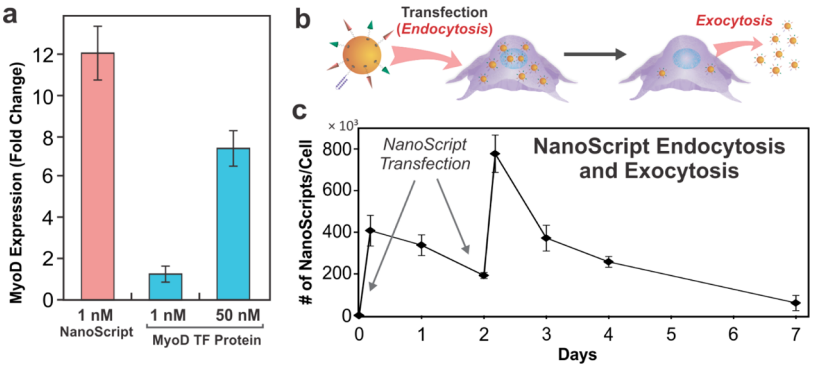


Figure 6. NanoScript mimics the gene-regulating function TF proteins and is excreted after inducing differentiation. (a) MyoD-TF protein was delivered using commercial transfection kits, and after 7 days, the gene expression of *MyoD* was evaluated. At a similar concentration (1 nM = 10 μg/mL), NanoScript-MRF triggers moderately higher levels of *MyoD* expression. The MyoD expression induced by NanoScript is identical to that in Figure 5a. (b) Schematic representation of NanoScript being taken up by cells through a mechanism called endocytosis and NanoScript being cleared from cells through a mechanism called exocytosis. (c) NanoScript-MRF was transfected on day 0 and day 2, and the gold content within the cells was quantified at various time points using inductively coupled plasma optical emission spectrometry (ICP-OES). The number of NanoScript particles remaining inside the cell was determined by quantifying the number of gold nanoparticles using a standard curve and then dividing by the number of cells. Based on microscope visualization, the cells stopped proliferating during the differentiation period, which is a phenomenon supported by literature.[43] The sharp peaks at day 0 and day 2 correlate to the delivery of NanoScript, and approximately 95% of NanoScript-MRF is cleared by day 7 (calculated by dividing the number of particles on day 7 by the highest peak on day 2). Error bars are from three independent experiments.

effects on the DNA, such as inducing double-strand breaks,[37] using a commercially available staining kit, and we found that NanoScript does not generate double-stand breaks (Supporting Information Figure S8). Collectively, these findings establish that genetic reprogramming by NanoScript-MRF can initiate transcriptional activity of differentiation-specific genes in stem cells to generate muscle cells.

NanoScript-MRF Mimics the Functions of Natural Transcription Factors. Next, to validate our claim that NanoScript emulates the function of natural TFs, we compared our NanoScript to commercially available TF proteins, such as the *MyoD*-TF, which functions to regulate myogenesis. We transfected various concentrations of the *MyoD*-TF protein into ADMSCs following the protocol from a commercially available protein delivery kit, which is based upon conventional cationic-based lipids for transfection. After 7 days, we observed a dose-dependent increase of *MyoD* gene expression (Figure 6a); however, the expression was less than that induced by our NanoScript-MRF at a similar concentration. This result suggests that NanoScript effectively mimics the gene-regulating function of conventional TF proteins.

NanoScript Is Naturally Cleared from Cells. Finally, a strongly desired characteristic of differentiated cells is that they are free of any foreign or exogenously introduced materials. The movement of foreign cargo across the cell membrane is regulated by natural mechanisms called endocytosis and exocytosis (Figure 6b). We wanted to ensure that the generated muscle cells did not contain any inorganic remnants from the gold nanoparticle core of NanoScript. To this end, we monitored the gold content in the cells throughout

the differentiation period. Immediately after treatment of NanoScript, there was a significant increase in the number of NanoScript particles inside the cell, but by the end of the differentiation period on day 7, most of the NanoScript particles (~95%) were excreted from the cells (Figure 6c). This result as in agreement with previous reports that demonstrate exocytosis of gold nanoparticles.[24,38] As a result of almost all gold remnants being excreted from the differentiated muscle cells, we believe that the NanoScript platform can be effectively utilized for stem cell applications.

CONCLUSIONS

In conclusion, we have successfully demonstrated that a completely artificial, nanoparticle-based transcription factor called NanoScript can be designed to replicate the function of differentiation-specific TFs. Specifically, we developed a NanoScript specific for MRFs to induce muscle differentiation. NanoScript-MRF is stable in physiological environments, small enough to localize within the nucleus, efficient in targeting and activating MRF genes, potent enough to induce muscle differentiation of ADMSCs, and is safely cleared from differentiated cells. Moreover, considering that direct TF delivery is hindered by low transfection efficiency,[39] our NanoScript-MRF mimicking the functions of natural TFs could be an alternative method for stem-cell-based regenerative medicine. Compared to natural TFs, our NanoScript platform has several distinct advantages, including the ability to mimic any TF protein by simply tuning one small molecule component; the multifunctional nanoparticle core can potentially be used for simultaneous imaging and tracking and the ability to localize with the nucleus due to a specific membrane-penetrating peptide, all of which leads to enhanced gene expression in a nonviral manner.

As the NanoScript platform regulates genes in a nonintegrative approach, it is a highly attractive tool for conventional gene-regulating methods. As such, we envision that the multifunctional surface of NanoScript will allow for conjugation of other small molecules that can further enhance the gene-regulating capabilities of NanoScript and expand its scope of being utilized for stem cell applications. For example, addition of small molecule gene regulators, such as RNAi and oligonucleotides,[40,41] or epigenetic modification molecules[42] onto NanoScript can potentially enhance its gene expression capabilities. Furthermore, by modifying the hairpin polyamide sequence, NanoScript can easily be designed to target and activate almost any gene of interest, including differentiation-specific genes. Finally, the current gold nanoparticle core can easily be changed to another type, such as magnetic core–shell nanoparticles or mesoporous silica nanoparticles, which can enable real-time *in vivo* tracking using magnetic resonance imaging and multifaceted delivery, respectively, without compromising the overall concept and design of NanoScript. As a result of these unique and remarkable features, we strongly believe that NanoScript represents a powerful tool to replicate almost any TF for gene-regulating applications, including stem cell differentiation and cellular reprogramming.

METHODS

Construction of NanoScript. The NanoScript platform was constructed using a two-step method. First, the amine-terminated biomolecules were conjugated to a linker molecule, SH-PEG-COOH [thiol-PEG-carboxy 1kDa (Creative PEGWorks, PBL-8073)]. The PEG molecule was dissolved into a 50 mM ethanol solution. Then, 50 mM of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide) (EDC) (Sigma) and 50 mM of *N*-hydroxysuccinimide (NHS) (Acros Organics) was added to this solution and then placed on a shaker for 1 h to activate the carboxyl group. Afterward, a 5 mM solution of the three biomolecules (MRF-DBD, TAP, and CPP) was added to the solution and was allowed to react at room temperature for 2 h. A solution containing 10 molar excess of PEG-MRF-DBD, PEG-TAP, and PEG-CPP with a mole ratio of 2:2:1 was added dropwise to the 10 nm AuNP solution and allowed to stir for 2 h. The functionalized AuNPs (termed NanoScript) were filtered three times using a 10 000 MCWO filter (Millipore) to remove unreacted molecules and to adjust the volume and concentration. The dye-labeled NanoScript, used for tracking intracellular localization of NanoScript, was constructed by conjugating the Alexa Fluor 594 (Invitrogen) fluorescent dye to TAP. Specifically, the free carboxy group on PEG-TAP was further conjugated to the Alexa Fluor 594 hydrazide dye *via* EDC/NHS coupling as described above.

ADMSC Culture and Myogenic Differentiation. The human ADMSCs (American CryoStem) and the 0.5% fetal bovine serum growth media were provided by American CryoStem. All cell culture was performed using the manufacture's protocol. The ADMSCs were grown on fibronectin-coated (2 μg/cm^2, Millipore) culture dishes and maintained at 37 °C in a humidified incubator with 5% CO_2. For consistency, all experiments were carried out on cells between passages 2 and 4. For myogenic differentiation experiments, ADMSCs were seeded in a 24-well plate at a density of 20 000 cells per well. After 24 h, 10 μg/mL of NanoScript-MRF was incubated with the cells for 4 h. Thereafter, the cells were washed twice with PBS (phosphate-buffered saline), and myogenic medium was added. The myogenic growth media comprised 2% horse serum (Sigma), human transferrin (2.5 μg/mL, Sigma), human insulin (5 μg/mL, Life Technologies), and 1% penicillin and streptomycin (Life Technologies) in high glucose DMEM (Dulbecco's modified Eagle medium, Invitrogen). A 10 μg/mL of NanoScript-MRF was transfected on day 2 for 4 h and washed twice with PBS, and then fresh myogenic medium was added. The cells were allowed to differentiate for 7 days in myogenic growth medium, which was replaced with fresh medium every other day. The MyoD-TF protein (Abcam, #ab134857) was transfected into ADMSCs on day 0 and day 2 using a commercially available kit (Thermo Scientific, Pro-Ject Protein Transfection Reagent #89850) and by following the manufacturer's protocol.

Immunocytochemistry. To investigate the nuclear localization of the dye-labeled NanoScript in ADMSCs, the medium was removed and the cells were fixed for 15 min in formalin (Sigma) followed by two washes with PBS. The nucleus was stained with DAPI (Life Technologies) for 30 min and then washed with PBS three times. To investigate the extent of myogenic differentiation,

on day 7, the cells were fixed with formalin for 15 min and then washed twice with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min, and nonspecific binding was blocked with 5% normal goat serum (NGS, Life Technologies) in PBS for 1 h at room temperature. To study the extent of myogenic differentiation, the primary mouse antibody against myosin (1:200 dilution, Sigma) and the primary rabbit antibody against myogenin (1:200 dilution, Santa Cruz Biotechnology) was used. Following the manufacturer's protocol, the fixed samples were incubated overnight at 4 °C in a solution of these antibodies in PBS containing 10% NGS. After being washed three times with PBS, the samples were incubated for 1 h at room temperature in a solution of anti-mouse secondary antibody labeled with Alexa Fluor 647 (1:100, Life Technologies), anti-rabbit secondary antibody labeled with Alexa Fluor 546 (1:100, Life Technologies), and DAPI (1:100, Life Technologies) in PBS containing 10% NGS. After being washed three times, all samples were imaged using a Nikon T2500 inverted fluorescence microscope.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Experimental details of MRF promoter sequence, polyamide binding affinity, stability in aqueous enviornments, nuclear localization, myogeneic controls, and primer sequnces used for PCR analysis. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.5b00709.

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