

SUPPORTING INFORMATION

Inducing Stem Cell Myogenesis using NanoScript

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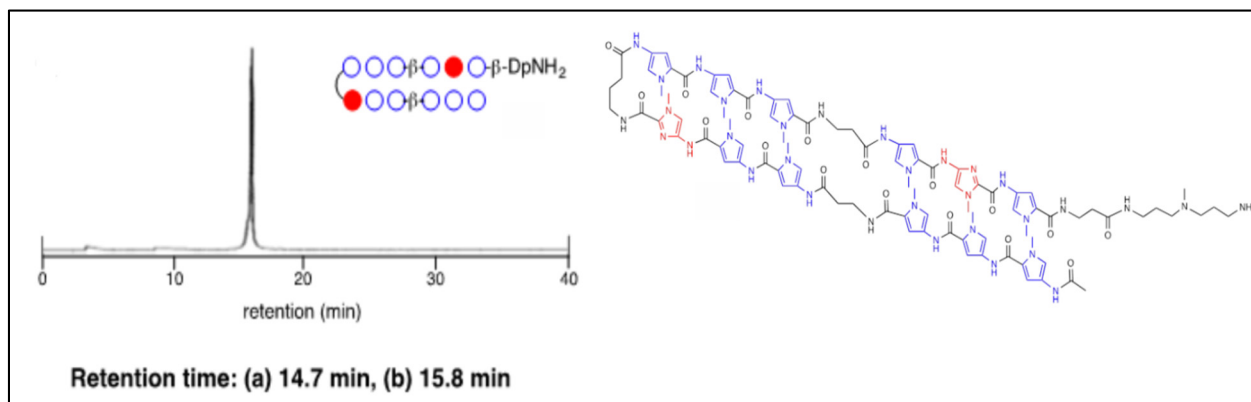
MATERIALS AND METHODS

Synthesis of Synthetic Transcription Factors (STFs): The transactivation peptide, having a sequence of CGSDALDDFDLDMLGSDALDDFDLDMLGS-NH₂ in the D-form, was purchased from GenScript. The cell penetrating peptide (CPP), having a sequence of CALNNAGRKKRRRQRRR-OH, was purchased from GenScript.

The synthesis of the polyamide was performed as previously reported.¹ All machine-assisted Py-Im polyamide synthesis was performed using a PSSM-8 peptide synthesizer (Shimadzu, Kyoto) with a computer-assisted operation system at 40 mg of Fmoc-β-Ala-Wang resin (ca. 0.55 mmol/g, 100~200 mesh, Novabiochem) by using Fmoc chemistry. The reaction steps underwent the following synthetic cycle: i) deblocking steps for 4 min x 2, 20% piperidine in DMF; ii) coupling step for 60 min, corresponding carboxylic acids, 1H-Benzotriazolium, 1-[bis(dimethylamino)methylene]-5chloro-hexafluorophosphate (1-),3-oxide (HCTU) (88 mg), diisopropylethylamine (DIEA) (36 μL); iii) washing steps for 1 min x 5, DMF.

In the coupling step, each of the corresponding carboxylic acids were prepared in a 1-methyl-2-pyrrolidone solution of Fmoc-Py-COOH (77 mg), Fmoc-Im-COOH (77 mg), Fmoc-PyIm-COOH (100 mg), Fmoc-γ-COOH (69 mg). All couplings were carried out with stirring by N₂ gas bubbling. All lines were washed with DMF after solution transfers. After the completion of the synthesis by the last acetyl capping on the peptide synthesizer, the resin was washed with DMF and methanol, and dried in a desiccator at room temperature in vacuo. For 3 h at 55 °C, a dried resin was cleaved with 0.4 ml of 3,3'-diamino-N-methyldipropylamine. Then the reaction mixture was filtered, triturated from CH₂Cl₂ and Et₂O. This yielded a crude yellow powder. Purification was performed by flash column chromatography (elution with 0.1% trifluoroacetic acid in water and a 0-35% acetonitrile linear gradient (0-35 min) at a flow rate of 18 mL/min⁻¹, 254 nm).

Analytical HPLC: Retention Time = 15.8 min (0.1 % TFA in water with CH₃CN as eluent, and a linear gradient elution of 0-100% CH₃CN over 40 min). ¹H NMR (600 MHz, [D₆]DMSO); 10.26 (s, 1H; NH), 10.21 (s, 1H; NH), 9.99 (s, 1H; NH), 9.92 (s, 1H; NH), 9.90 (s, 1H; NH), 9.88 (s, 4H; NH), 9.83 (s, 1H; NH), 9.81 (s, 1H; NH), 9.09-9.04 (m, 6H; NH), 7.53 (s, 1H; CH), 7.49 (s, 1H; CH), 7.31 (s, 1H; CH), 7.27 (s, 1H; CH), 7.22-7.19 (m, 4H; CH), 7.17 (s, 2H; CH), 7.16 (s, 1H; CH), 7.14 (s, 1H; CH), 7.09 (m, 2H; CH), 7.04 (m, 2H; CH), 7.00 (s, 2H; CH), 6.97 (s, 1H; CH), 6.94 (s, 1H; CH), 6.86-6.85 (m, 2H; CH), 3.96 (s, 3H; NCH₃), 3.93 (s, 3H; NCH₃), 3.84-3.80 (m, 33H; NCH₃), 2.74-2.73 (d, J = 4.8 Hz, 6H; CH₂), 2.62 (m, 4H; CH₂), 2.38-2.35 (m, 6H; CH₂), 2.29-2.27 (t, J = 7.8 Hz, 4H; CH₂), 1.97 (s, 3H; COCH₃), 1.91-1.86 (m, 4H; CH₂), 1.81-1.76 (m, 6H; CH₂). ESI-TOF-MS (positive) m/z calcd for C₉₂H₁₁₅N₃₃O₁₇+ [M+2H]²⁺+976.96; found 976.94.



Synthesis of Gold Nanoparticles: The gold nanoparticles with an approximate diameter of 10 nm were prepared by the Ferns method of citrate reduction of H_{Au}Cl₄ following establish protocols and a previous report. First, the glassware was cleaned in aqua regia (3 HCl: 1 HNO₃, handle with extreme caution!), and oven dried after washing with water. While stirring, a 100 mL aqueous solution of 1mM H_{Au}Cl₄ was heated to a reflux. Then 16 mL of 1% (by weight)

sodium citrate was quickly added, resulting in a change in solution color from yellow to ruby red. After the solution turned ruby red, the solution was refluxed for another 5 minutes, then cooled to room temperature and filtered using a 0.45 μm syringe filter.

NanoScript Characterization: Characterization of the AuNPs was performed using dynamic light scattering (DLS) and the concentration was obtained using UV-vis spectroscopy. The gold nanoparticle concentration was obtained using UV-visible absorption spectra (Varian Cary 5000 UV Vis-NIR Spectrophotometer). Second, using Dynamic Light Scattering (Malvern Zetasizer Nano-ZS90), we measured the hydrodynamic size and zeta potential (surface charge) of NanoScript.

SPR Binding Affinity: The SPR assays were performed using a BIACORE X instrument. Biotinylated hairpin DNAs were purchased from JBioS (Tokyo, Japan) and the sequences are shown in Figure 2. The hairpin biotinylated DNA was immobilized to streptavidin-coated sensor chip SA to obtain the desired immobilization level (approximately 900 RU rise). SPR assays were carried out using HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005 % Surfactant P20) with 0.1 % DMSO at 25 °C. A series of sample solutions with various concentrations were prepared in the buffer with 0.1 % DMSO and injected at a flow rate of 20 $\mu\text{l}/\text{min}$. To measure the rates of association (k_a), dissociation (k_d) and dissociation constant (KD), data processing was performed with an appropriate fitting model using the BIAevaluation 4.1 program. The 1:1 binding with mass transfer was used for fitting the sensorgrams.

ICP-OES Measurement: After ADMSCs were seeded in a 24-well plate (20,000 cells/well), 10 $\mu\text{g}/\text{mL}$ of NanoScript was added on Day 0 and 2 as per the differentiation timeline. At various

time points, the cells were washed with PBS and detached using TrypLe (Gibco). After centrifugation, the supernatant was removed, and the cells were re-suspended in PBS and counted using a hemocytometer. After centrifugation, the supernatant was removed and 25 μ L of lysis buffer was added. Then 250 μ L of aqua regia (caution: extremely reactive) was added and allowed to sit overnight in an eppendorf tube (Note: Immediately after closing the cap of the eppendorf tube, puncture a small hole in the cap to prevent pressure buildup within the tube). The next day, the solution was transferred to 9.725 mL of distilled water (10 mL total volume with 2.5 % aqua regia). Cellular uptake experiments were performed three times and each replicate was measured for gold content three times by Inductively coupled plasma optical emission spectroscopy (Perkin Elmer Optima 7300 DV), operating under normal conditions. A standard curve comparing the number of gold nanoparticles to the Au intensity was established using known nanoparticle concentrations.

Evaluating Double Strand Breaks: Adopting a protocol from a previous report,² we purchased a specific staining kit (OxiSelect™ DNA Double Strand Break Staining Kit, Cell Biolabs). We followed the manufacturer's staining protocol and imaged the cells using a Nikon T2500 inverted fluorescence microscope.

HPLC To Quantify Ligand Amount: In order to quantify the amount of ligands on the gold nanoparticle (AuNP) surface, we performed HPLC (Agilent LC 1100) using a Zorbax Extend-C18 Solvent Saver Plus, 3.5 μ m, 3.0 \times 150 mm, column. The thiol-terminated molecule solution before and after conjugation was analyzed, and using a standard curve, we were able to calculate the

amount remaining on the nanoparticle. Furthermore, the amount of gold nanoparticles was calculated using its excitation coefficient.

PCR Analysis: Total RNA was extracted with TRIzol reagent (Invitrogen) and was reverse transcribed to cDNA with Superscript III Reverse Transcriptase (Invitrogen). Conventional quantitative RT-PCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-time PCR System (Applied Biosystems) following the manufacturers protocol. Primers sequences for the genes can be found in **Table S1**.

SUPPORTING FIGURES AND TABLES

<p>MyoD CCTCAGCTCTGTGCCCCCAATGCCTTGCCTCTCTCCAAATCTCTCACGACCTGATTTCTACAGCCGCTCTACCCATGGG TCCCCACAAATCAGGGGACAGAGGAGTATTGAAAGTCAGCTCAGAGGTGAGCGCGCGCAGCCAGCGTTTCCCGCGGAT ACAGCAGTCGGGTGTTGGAGAGGTTTGGAAAGGGCGTGCCGGAGAGC<u>CAAGTG</u>CAGCCGCCTAGGGCTGCCGGTCGCT CCCTCCCTCCCTGCCCGGTAGGGGACCTAGCGCGCACGCCAGTGTGGAGGGGCGGGCTGGCTGGCCAGTCTGCGGGCCC CTGCGGCCACCCCGGGGACCCCCCAAGCCCCGCCCGCAGTGTTCCTATTGGCCTCGGACTCCCCCTCCCC<u>CAGCTG</u> CCCGCCTGGGCTCCGGGGCGTTTAGGCTACTACGGATAAATAGCCCAGGGCGCCTGGCGAGAAGCTAGGGGTGAGGAAG CCCTGGGGCGCTGCCCGCCTTTCCTTA</p> <p>Myf5 TTCAAGTTTTTATAGTAAACCGTTATTCAATC<u>CAATTG</u>CTGGAAAAAGTTTTTCGAGATAATTGAAGTTCATGAAA AAGAGAAACAAACACCCCCAAAT<u>CAGGTG</u>ATTCCCTCACGCCCAGGATTGGGAGGGCAGGTTCGGCTCTTGGTG C<u>CAGCTG</u>AAAGGGATTCTATTTATTACCCTATTTTAAAATGTGTGTC<u>CAATTG</u>CAAAAAGTTGGACTGCC TTGGTCACTTTTGTGTGAAACACTCCTTAAGTGATCCCTGTTTTAGCCTATCTAGAGAGTG<u>CAAATG</u>GAGCA GGTTGGGGACCCACTGCCGCCAGCAGAAATACAGTAAAACCATTAGCGTCAAAAGGGGCA<u>GTACAC</u>GAGCA GATTGTCTGTAGAGGAAAGGCGGAGGTTTGTCCAGACAGCCCCCGCGGGGGTTGCGGTGGGATATGCTAATA GTGCCTGGCCACTGGGGCCGGCCTCCCTCCCAGAAAGTATATAAAGAACCCCAACCCAGCTTGTCT</p> <p>Mrf4 TGAAAGTCATGTATAACCTTTGAAGCACTGTAATGGTTTTAATCACAGGAATAGCATCTTTTTGAGAGGCAG GAGAGAGAAAGGAGCTGAACCACTCTGCAATATTAACCCGGCTATTTTGGAGCAATGGCTATTGCCCTTTGA AATCTTTCATCATGGGTCTATACTTAGAATT<u>CAGATG</u>AGCGCAGGGGGAGGGGAATAGGGCAATGTTAATCC C<u>CAGTTG</u>TTTTGAAGTTGTCCTGTAAGCAGGTTAGTATCTAACTGCCAAAATGGAAGGGTTCAATGCTTT GTTTAAGTTATGTGATTCTCTCTGTGTAACCTGGTCTGCACATAGATTGTCACAACTCTGGGAGGTGACA CTTCTCAGTTTTCTTTCAAACCTAGATGTTCTAGAGAGCACTAGCTGCAATCACATTGGGAGACTGATGCTCC ATGACAGCTAAAAGTTGATTGAGTTTCAGGAGCTACTATATATAAAGCTGG<u>GTCGAC</u>TTATGTCACT</p> <p>Myogenin CTCATCGACTGATGTAGTGTGGTTAAGGTGCTGTGAGGAAGCAAGGAGATGGATAAGTTGGCTCTTAAGGCC CCTTCCAGCCTAAGCCTACCCCTCCTTGTTCCTCCTCCCCCTATACCCACCAGCTTCTTCGGGACTGCAAG GAGACAGCAGGCAGGCCGCCAGCTAGGAGTAATTGAAAGGAG<u>CAGATG</u>AGAGGGGAATGTGTCCTCCCCCA ACGCCCTGCCCCACAGGGGCTGCTGAGAAATGAAAACCTAATCAAATTACACCCGACGGCCTCCCGACCC<u>GT</u> <u>GCAC</u>AGGAGCCCGCCTGGGCCAGGGCAGGCTGGCAGGGTGGGGTGGGGGCCATGCGGGAGAAAGAAGGGGA ATCACATCTAATCCACT<u>GTA AAC</u>GCCTTGATGTGAGCAACAGCTTAGAGGGGGCTCAGGTTTCTGTGGCGT TGGCTATATTTATCTCTGGTTCCATGCCAGCGGGGAGGGTTTAAATGGCACCCAG<u>CAGTTG</u>GCGTGAG</p>

Figure S1: MRF Promoter Sequences. For each gene, the first 500 base pairs directly upstream from the Transcription Start Site are shown. The consensus CANNTG (N = any base pair) sequences are underlined. Gene sequences were obtained from the online “Ensembl Genome Browser.”

Ligands/AuNP (10nm)
1297 ± 102

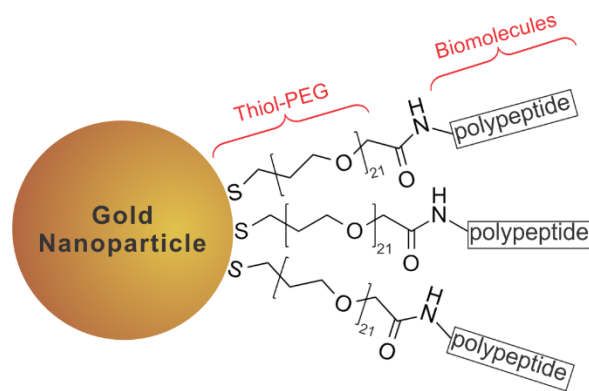


Figure S2: Ligand Amount per Nanoparticle. In order to calculate the ligand amount on the gold nanoparticle (AuNP), we performed HPLC. Results showed that the gold nanoparticle hold 1297 ± 102 ligands on its surface. This result is corroborated by a literature report that estimated a thiol-terminated ligand density of 4.3 ligands/nm² on gold nanoparticles, and hence 1350 ligands on a 10 nm gold nanoparticle.³ The ligands are thiol-PEG-peptide molecules which were functionalized on the nanoparticle via the thiol group.

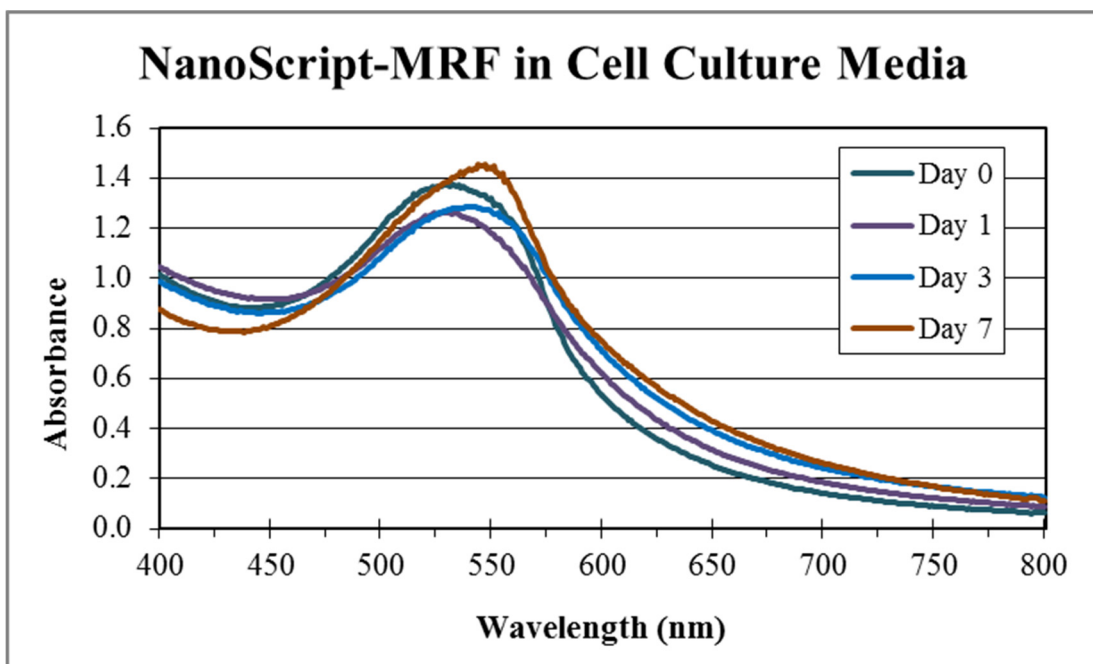


Figure S3: NanoScript-MRF is Stable in Physiological Environments. It is important that NanoScript-MRF remains mono-dispersed and is stable in physiological environments. To this end, NanoScript-MRF was incubated at 37°C in cell culture media. Evaluation of NanoScript-MRF absorbance at various time points reveals the overall absorbance profile to be mostly similar with only slight changes to the absorbance peak even after 7 days of incubation, thus suggesting that NanoScript-MRF is stable in physiological conditions.

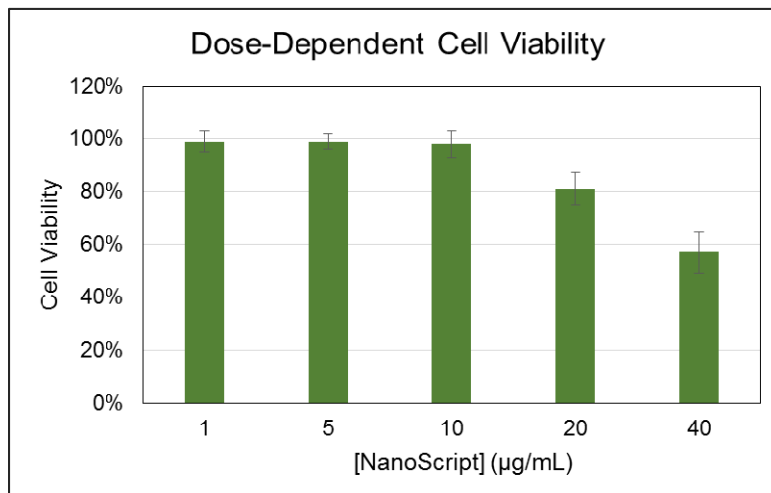


Figure S4: Dose-Dependent Cell Viability. In order to identify the optimal NanoScript concentration to keep cell viability high, we performed a dose-dependent cell viability MTS assay. Different concentration of NanoScripts (ranging from 1 – 40 µg/mL) were transfected into ADMSCs and after 3 days, their cell viability was evaluated. We found that the maximum NanoScript concentration of that maintains high viability is 10 µg/mL, and hence, we used this concentration for all subsequent experiments in this paper. Cell viability is relative to untreated cells and standard error is from three independent trials.

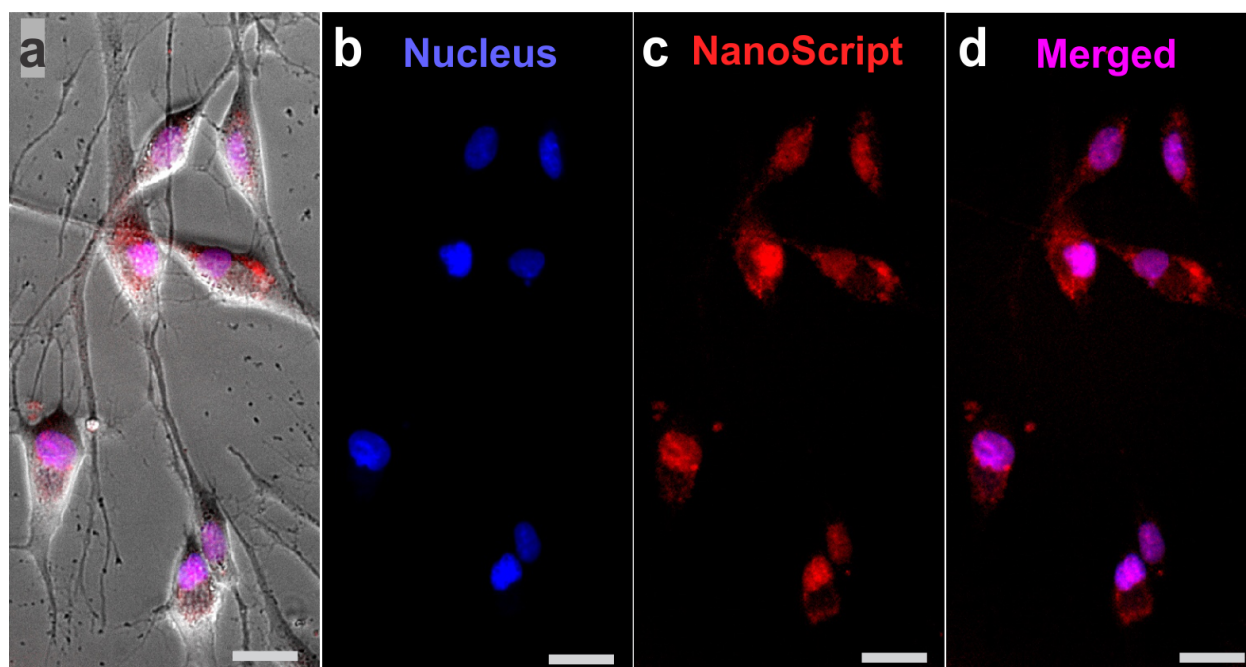


Figure S5: NanoScript Enters ADMSCs and Localizes in the Nucleus. (a-d) 48 hr post-transfection, fluorescence microscopy was used evaluate the intracellular localization of NanoScript. The merged image (a) shows the morphology of the ADMSCs with an overlay of the fluorescence images. The combination of the DAPI (blue) (b), the dye-labeled NanoScript-MRF (red) (c), and the merged fluorescence images (d), indicates that the NanoScript-MRF in uptaken and is localized inside the nucleus. (Scale bar = 20 μm)

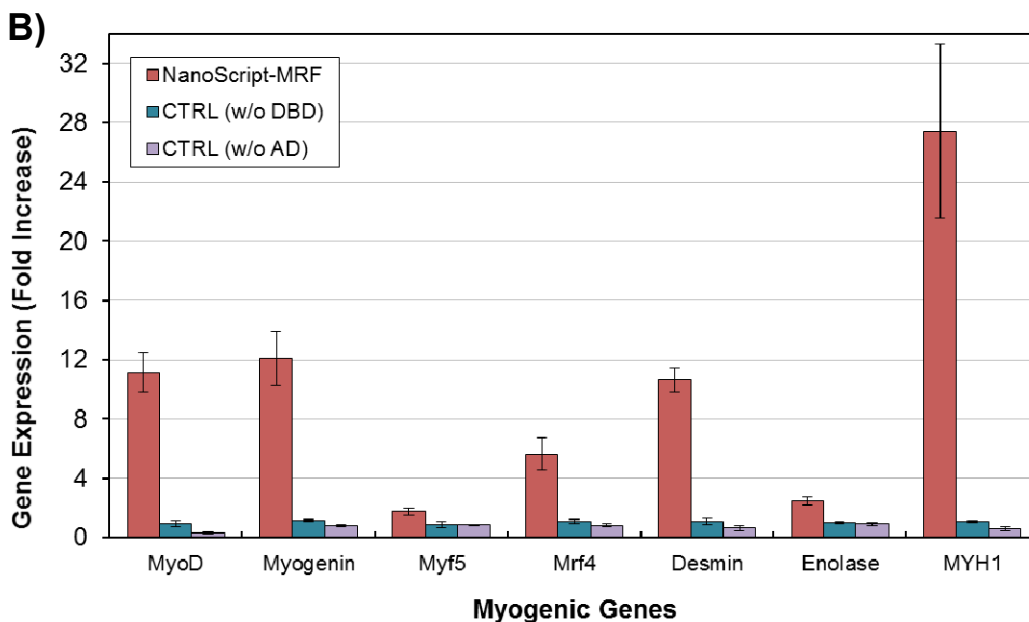
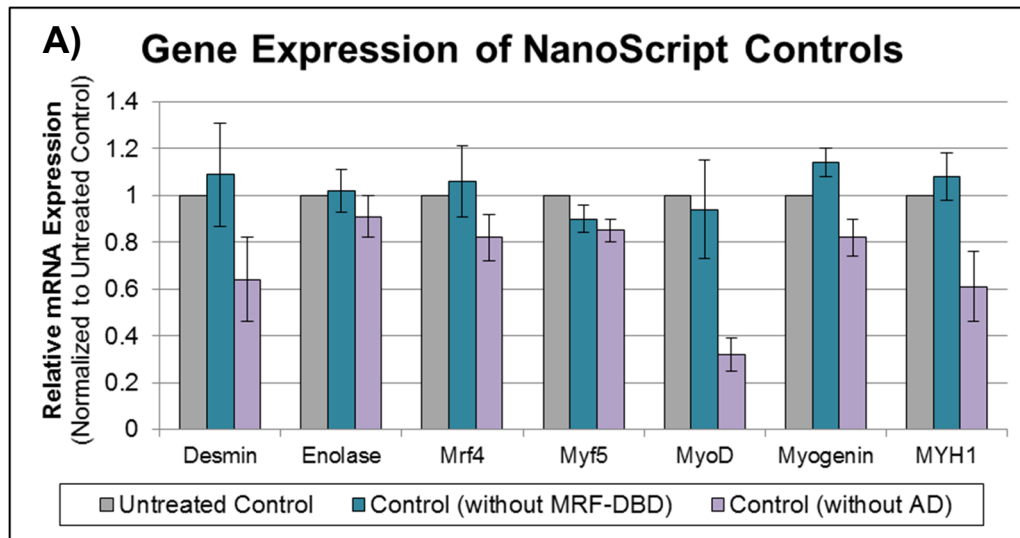


Figure S6: Gene Expression of NanoScript Controls. (a) Control experiments were performed to evaluate the importance of each domain on NanoScript. Specifically, NanoScript lacking individual domains, such as the AD and DBD, were tested on ADMSCs and the gene expression was quantified by qPCR. The same myogenic genes shown in Figure 6a were tested and showed negligible expression beyond basal levels. Error bars are from three independent trials and mRNA expression is represented as a fold change compared to the control. (b) The expression of each gene for all three conditions have been combined together on a single graph. Control conditions include NanoScript without either the DBD or AD molecules. Expression levels have been normalized to the untreated control.

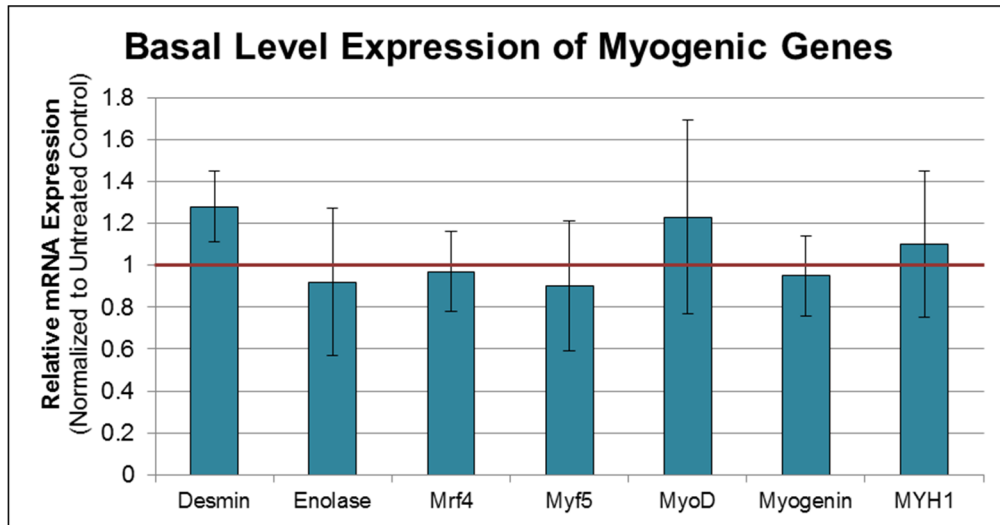


Figure S7: Basal Level Expression of Myogenic Genes. When the relative basal level expression of each gene was quantified, there was minimal change in the gene expression profile. Hence, by only incubating the cells with myogenic media, the basal gene expression is very minimal. The red line represents the normalized expression for each gene to the control.

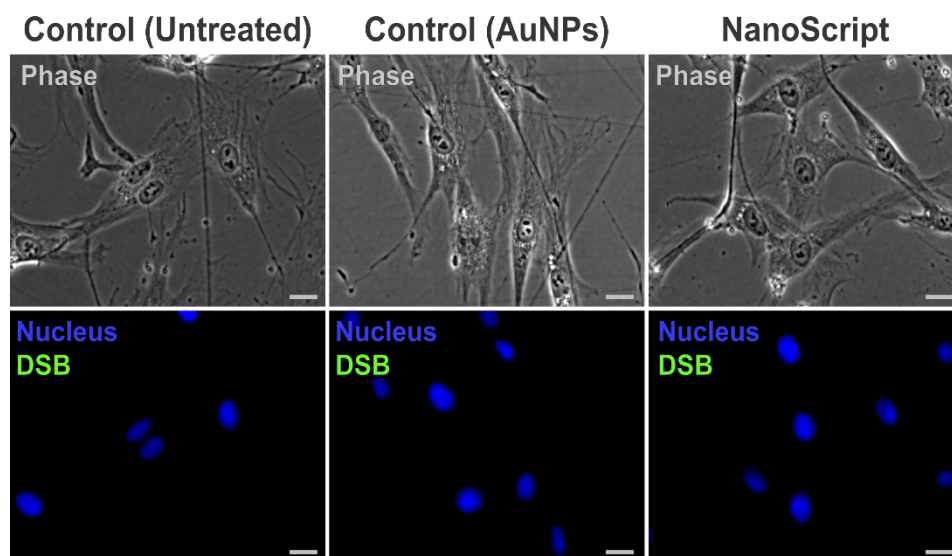


Figure S8: Evaluating Presence of Double Strand Breaks in the DNA. To test the presence of induced double strand breaks (DSBs) in the DNA by NanoScript, we performed immunostaining using a commercially-available kit. The immunostaining images did not detect the presence of DSBs (green, second row), either in the cytoplasm or nucleus (blue, second row), in the three conditions (Scale bar = 20 μ m).

Target	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Expected Size (bp)
Desmin	GAGACCATCGCGGCTAAGAAC	GTGTAGGACTGGATCTGGTGT	152
Enolase	GGCTGGTTACCCAGACAAGG	TCGTACTIONCCATTGCGATAGAA	75
MyoD	CGGACGTGCCTTCTGAGTC	AGCACCTGGTATATCGGGTTG	145
MRF4	GGAGCGCCATCAGCTATATTG	ATCCGCACCCTCAAGATTTTC	131
MYF5	CTGCCAGTTCTCACCTTCTGA	AACTCGTCCCCAAATTCACCC	78
Myogenin	TACCAGGAACCCCGCTTCTA	AGGTGGACAGGCAGGTAGTT	50
Myosin Heavy Chain I (MYH1)	CCCTACAAGTGGTTGCCAGTG	CTTCCCTGCGCCAGATTCTC	174
CD44	CTGCCGCTTTGCAGGTGTA	CATTGTGGGCAAGGTGCTATT	109
CD49D	CACAACACGCTGTTCGGCTA	CGATCCTGCATCTGTAAATCGC	139
CD90	ATCGCTCTCCTGCTAACAGTC	CTCGTACTGGATGGGTGAACT	135
CD105	TGCACTTGGCCTACAATTCCA	AGCTGCCCACTCAAGGATCT	107

TABLE S1: PRIMER SEQUENCES AND THE EXPECTED BAND SIZE.

KEY REFERENCES FOR SUPPORTING INFORMATION

1. Patel, S.; Jung, D.; Yin, P. T.; Carlton, P.; Yamamoto, M.; Bando, T.; Sugiyama, H.; Lee, K.-B. NanoScript: A Nanoparticle-Based Artificial Transcription Factor for Effective Gene Regulation. *ACS Nano* 2014, 8, 8959-8967.
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3. Hinterwirth, H.; Kappel, S.; Waitz, T.; Prohaska, T.; Lindner, W.; Lämmerhofer, M. Quantifying Thiol Ligand Density of Self-Assembled Monolayers on Gold Nanoparticles by Inductively Coupled Plasma–Mass Spectrometry. *ACS Nano* 2013, 7, 1129-1136.