NANOSCRIPT: A NANOPARTICLE-BASED BIOMIMETIC PLATFORM FOR STEM CELL REPROGRAMMING

BY: SAHISHNU V. PATEL

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Dr. Ki-Bum Lee
and approved by:

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ABSTRACT OF DISSERTATION

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By: SAHISHNU V. PATEL
Dissertation Director: Dr. Ki-Bum Lee

Stem cell engineering for regenerative medicine offers new hope for treating many ailments and injuries. Hence, there is an urgent demand by stem cell scientists for an alternative platform that induces stem cell differentiation in a safe and efficient manner. Stem cell differentiation is inherently regulated by transcription factors (TFs), which are multi-domain proteins that interact with DNA to control expression of target genes, and thus, TFs are master regulators of gene expression and cellular behavior. Recently, scientists have developed synthetic transcription factors (STFs), which are small molecules that mimic the function of the individual domains on TF proteins.

This work presents the development a novel bio-inspired platform called NanoScript, which is an alternative approach for safe stem cell differentiation. NanoScript is a nanoparticle-based artificial TF protein because it is designed to replicate the function and structure of natural TF proteins. NanoScript was constructed by assembling STFs onto
multifunctional nanoparticles. We first demonstrate that NanoScript localizes within the nucleus of cells, initiates transcription of a reporter plasmid by over 15-fold in cancer cells, and transcribes endogenous genes.

The tunable and interchangeable components of NanoScript can easily be modified to either activate or deactivate any gene of interest. As a result, NanoScript was then demonstrated for three stem cell-based applications: 1) NanoScript targets myogenic genes to differentiate adipose-derived mesenchymal stem cells (ADMSCs) into muscle cells, 2) NanoScript modified with an epigenetic modulator, CTB, increases transcriptional potency and enhances differentiation of ADMSCs into chondrocytes, and 3) NanoScript redesigned with gene repression molecules acts a transcriptional repressor protein because it downregulates gene expression to induce differentiation of neural stem cells into functional neurons.

Because of its robust tunability and biocompatibility, the patented NanoScript platform is a promising alternative tool for research scientists for applications involving gene manipulation such as stem cell differentiation, cancer therapy, and cellular reprogramming. Moreover, the ability of NanoScript to induce stem cell differentiation in a non-viral and footprint-free manner is highly desired by stem cell clinicians, and hence, holds potential for use in stem cell-based therapies.
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Chapter 1

Nanoparticle-Based Approaches for Stem Cell Biology

While several advances have been made in developing stem cell platforms and methods to induce their differentiation, there are two critical aspects need to be addressed: 1) efficient delivery of nucleic acids and small molecules for stem cell differentiation, and 2) effective, non-invasive, and real-time tracking of transplanted stem cells. To address this, there is an increasing trend of utilizing various types of nanoparticles to not only deliver biomolecules to targeted site, but also to track the location of transplanted stem cells in real-time. Over the past decade, various types of nanoparticles including, magnetic nanoparticles, silica nanoparticles, quantum dots, and gold nanoparticles, have been developed to serve as vehicles for targeted delivery. Finally, the development and limitations of small molecules called synthetic transcription factors (STFs) are described.
1.1 INTRODUCTION

Since the last 15 years, there has been significant progress in the field of stem cell biology, and as a result, patients suffering from terminal diseases or traumatic injuries have new hope for a potential therapy.\textsuperscript{1} The field of stem cell biology and stem cell-based regenerative medicine has been rapidly advancing as a promising therapy to treat debilitating diseases and injuries caused by the loss of terminal cells.\textsuperscript{3,6} It is because stem cells are known for their potential to repair and/or replace damaged tissue.

Stem cells are undifferentiated and multipotent cells that can differentiate into specialized cells based on intrinsic or external cues that manipulate their genetic code. Differentiation of stem cells into specific lineages relies on expression patterns of specific genes. In normal human development, stem cell differentiation is innately guided by expression of intrinsic cues. But forced stem cell differentiation to selectively control fate requires external cues such as a specific microenvironment or delivery of differentiation inducing factors.

It has long been vision for scientists to control stem cell behavior and fate as required for various clinical applications, and several methods to externally regulate stem cell fate have been developed. The long term goal is to harvest stem cells from patients, and through the use of various external cues, to generate specialized cells for implantation back into the patients. Even though progress have been made, the use of conventional methods to induce
differentiation such as viral vectors, DNA plasmids, small molecules, and a combination of thereof, have specific limitations. Hence, researchers been exploring alternatives, and as a result, the field of nanotechnology has significantly advanced for biological applications.

Nanotechnology has recently emerged as an exciting field of research involving the use of nanoscale materials for various applications including stem cell biology. Due to the extremely small scale of nanotechnology, ranging from 1-1000 nm, the potential of nanotechnology-based applications seems limitless. Researchers from multi-disciplinary fields have integrated expertise from inorganic chemistry, organic chemistry, material science, engineering, and stem cell biology to develop various nano-platforms and devices for manipulating stem cell behavior. In fact, over the past 10 years, the number of publications involving nanotechnology and stem cell biology has grown exponentially. This is the because of the great potential that stems from its amazing intrinsic qualities and widespread application potential.

There are two primary modes through which nanotechnology can regulate stem cell fate: 1) fabrication of nanoscale surfaces to mimic the various natural 3D microenvironment of cells, and 2) delivery of nanoscale materials to selectively target intracellular pathways. The cellular microenvironment in the body is a 3D dynamic process that cannot be effectively replicated in the traditional cell culture dishes. However, various nanoscale scaffolds and nanopatterned substrates with variable surface roughness and porosity have been fabricated to more effectively replicate the in vivo niche. As a
result, this not only provides insight into mechanistic studies to probe stem cell signaling pathways that induce differentiation, but also a novel method to induce differentiation by mimicking the microenvironment. Moreover, in an alternative approach, researchers have developed nanomaterials that can be used as intracellular deliver vehicles to introduce specific small molecules and biomolecules into cells. The small molecules that are delivered can selectively activate and regulate specific signaling pathways in stem cells to induce targeted differentiation. These nanomaterials can be of different shapes, sizes, and compositions, and thus they can be tuned for specific applications.

While several types of nanomaterials have been developed as deliver agents, the most prominent and widely used are nanoparticles. Nanoparticles are small, spherical materials that can range in size from 2-500 nm, and the most widely used types of inorganic nanoparticles include magnetic nanoparticles, gold nanoparticles, silica nanoparticles, and quantum dots (Figure 1.1). Each of these nanoparticles not only have the ability to carry specific small molecules into the cells, but have multifunctional properties such as contrast imaging, surface porosity, and magnetic capabilities for a synergistic effect to track and regulate stem cell behavior. Moreover, these nanoparticles are generally bio-compatible with minimal side effect or cytotoxicity, thus allowing them to be used as safe delivery agents. Because certain compositions of gold nanoparticles and magnetic nanoparticles have been approved by the FDA for clinical applications, there is a surge of using these nanoparticles for stem cell differentiation with potential for translation into the clinic.
Moreover, in a different field of research, scientists have developed specific biomolecules to supplement nanoparticles. Transcription factor (TF) proteins are master regulators of gene expression that participate in regulating transcriptional activity. TFs function to activate specific genes to alter cellular function and fate, and are critical elements that orchestrate the entire stem cell differentiation and cellular reprogramming process.\textsuperscript{10, 11} The molecular structure of TFs can be dissected into two critical domains: i) a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Designing Nanoparticles for Stem Cell Applications. The physical properties of nanoparticles can be selectively designed for specific applications based on the material composition and physical properties. Nanoparticles can be made functionally active depending on the surface chemistry and the functional biomolecules. Nanoparticles are highly tunable and have a modular chemistry, thus enabling their application for desired stem cell applications.}
\end{figure}
DNA-binding domain (DBD) that selectively targets and binds to specific sequences in the genome and ii) activation domains (ADs), which function by recruiting components of the transcription complex including RNA polymerase, mediators, and other TFs to initiate and sustain transcription. Due to the fact that natural TFs are comprised of components with independent functions (e.g. DBD and AD), it is possible to engineer small molecules called synthetic transcription factors (STFs) to target specific genes of interest and to regulate transcriptional activity in a similar manner as natural TFs. The exogenous delivery of STFs for the regulation of key signaling pathways is an attractive alternative for cellular reprogramming applications over current methods which rely on the delivery of plasmids or viruses. By using a combination of peptides and small molecules to mimic these functional domains, it has been demonstrated that STFs can bind to specific DNA sequences, initiate transcription, and subsequently induce target gene expression.

1.2 NANOPARTICLE UPTAKE MECHANISM

The first step for utilizing nanoparticle-based delivery of small molecules and exploiting their multifunctional properties for stem cells application involves efficient uptake of nanoparticles into the cell. Stem cells tightly regulate movement of cargo through the plasma membrane, and hence, a major challenge is the efficient intracellular uptake of nanoparticles. Because nanoparticles are comprised of inorganic compounds, the cell does not readily allow them to enter. However, the cell has multiple mechanisms to
allow large substances and cargo, such as nanoparticles, to enter through a mechanism called endocytosis. Endocytosis is an energy-dependent process by which cells engulf substances on the cell surface and shuttle them into the cytoplasm. Specifically, endocytosis is divided into four categories with slightly different mechanisms to shuttle different-sized foreign substances into the cell: clathrin-mediated endocytosis, caveolae, micropinocytosis, and phagocytosis. Depending on the size and surface makers of the nanoparticle, one of these mechanisms allows nanoparticle uptake into the cell (Figure 1.2).20

To facilitate the process of nanoparticle endocytosis, it is critical for nanoparticles to be functionalized with surface ligands that can bind to specific cell surface receptors. Cellular uptake dynamics for nanoparticle uptake is greatly dependent on the size of the nanoparticle and the peptides present on the surface. Cell penetrating peptides are a class of peptides that are specifically designed and conjugated on nanoparticles to enable plasma membrane penetration.22, 23 With the use of cell penetrating peptides and nuclear localization signal peptides, it is possible to engineer nanoparticles to not only cross the plasma membrane, but also the nuclear membrane to gain entry inside the nucleus.24 Moreover the size of nanoparticles has been demonstrated to play a significant role in both plasma and nuclear membrane uptake. Studies have shown that nanoparticles ranging from 10-100 nm can enter the cell, with an optimal nanoparticle diameter of 50 nm for maximum uptake. Therefore, depending on the application, the size of the nanoparticles
must be tuned. Furthermore, the chemical properties of ligands present on the nanoparticle surface also play a critical role in cellular uptake. Properties such as solubility, pH, and hydrophobicity influence cellular uptake.\textsuperscript{27}
When nanoparticles are incubated in biological fluid, such as cell culture media, proteins bind to the ligands on the nanoparticle surface to form what is called a protein corona, which can critically influence the interaction of nanoparticles with cells. The protein corona has been recently demonstrated to play a striking role in nanoparticle uptake. The concept of protein corona suggests that the mix of over 300 proteins rapidly adsorb on the nanoparticle surface within a few minutes, and the interaction of these proteins with the cell surface enable uptake. Even though several membrane penetrating peptides have been developed and demonstrated to be efficient enable intracellular localization of nanoparticle, the formation of the protein corona and its exact mechanism, which is still unclear, may play a significant role.

1.3 MAGNETIC NANOPARTICLES IN STEM CELL BIOLOGY

Magnetic nanoparticles (MNPs) are a class of nanoparticles, comprised of magnetic materials such as iron, cobalt, nickel, and zinc, which can be manipulated with a magnetic field. MNPs are spherical in shape and range in size from 10-100 nm, and their composition can have a mixture of various metals, such as Fe₃O₄, Fe₂O₃, NiFe₂O₄, and FeCo, that result in different magnetic properties. MNPs have desirable physiochemical properties, biological inertness, high stability in physiological conditions, and excellent magnetic properties that allow for non-invasive imaging and enhanced cellular uptake, thus establishing MNPs as excellent carriers of small molecules and biomolecules.
primary advantage of MNPs is their unique magnetic properties, and therefore, researchers have exploited these features by employing MNPs for three specific applications in stem cell biology: 1) enhanced delivery due to magnetofection (magnet-facilitated delivery), 2) stem cell tracking using various imaging techniques, \(^{35,37}\) and 3) magnetically guiding stem cells to targeted sites \textit{in vivo}.

### 1.3.1 Magnetically Facilitated Delivery for Enhanced Stem Cell Differentiation

Traditional methods to deliver MNPs, typically require cell penetrating peptides present the nanoparticle surface or transfection reagents; but increasing the transfection efficiency so that more MNPs enter the cell without compromising viability is a challenge. The unique magnetic properties of MNP enables enhanced delivery due to magnetofection, a technique which involves incubating MNPs in cell culture and placing a magnet underneath to generate a magnetic field that ‘pulls down’ the MNPs onto the cell surface.\(^{38}\) As a result, significantly more MNPs enter the cell and cell viability is not compromised through this process.

Recently, Lee and coworkers used a magnetofection-based approach to efficiently delivery MNPs into neural stem cells to induce neuronal differentiation.\(^{40-42}\) They used zinc-doped MNPs, ZnFe2O4, as a core and synthesized a gold shell to develop a magnetic core-shell nanoparticle (MCNP) (\textbf{Figure 1.3a, b, c}). The purpose of the outer gold shell is to increase biocompatibility, enable multiple biomolecules to anchor onto a single
nanoparticle, and prevent free radical formation from the MNP core. Specifically, the MCNPs were functionalized with a linker molecule to increase water solubility. Then the polyamine was coated to make the surface positive charged and then a small molecule nucleic acid called siRNA, which represses gene expression, was electrostatically

Figure 1.3: Magnetic Core-Shell Nanoparticles (MCNPs) for Stem Cell Differentiation and Imaging. A) Schematic of MCNPs functionalized with mercaptoundecanoic acid (MUA) followed by electrostatic conjugation of polyamide and nucleic acids for regulating gene expression in stem cells. B) A representative image showing that MCNPs with a composition of ZnFe₂O₄ are attracted to a magnet. C) Tem image of MCNPs (scale bar = 10 nm). D) MCNPs were incubated in GFP-labeled rat neural stem cells (rNSCs) and exposed to magnetofection (MF). The resulting GFP knockdown was quantified and is directly correlated to the gene-regulating efficiency of the MCNPs. The greater the GFP knockdown the greater its effect. E) Schematic of rNSCs undergoing magnetofection with MCNPs coated with nucleic acids targeting specific stem cell differentiation. F) Immunofluorescence images showing the differentiation into neuro-specific lineages with particular markers, TUJ1 (neuronal), GFAP (glial cells), and MBP (oligodendrocytes), based on the type of nucleic acid delivered.
conjugated on the surface (Figure 1.3a). First, delivery properties were tested using magnetofection, which revealed that GFP-labeled neural stem cells (NSCs) exposed to a magnetic for just 30 minutes significantly increased the transfection efficiency. Moreover, magnetofection-based delivery of MCNP showed a remarkable down-regulation in GFP as compared to MCNP delivered using conventional methods (Figure 1.3d). Then the MCNP was utilized for the delivery of siRNA to control neuronal differentiation of NSCs, which revealed that NSCs can be selectively differentiated to either neurons or oligodendrocytes in an efficient and non-toxic manner (Figure 1.3e, f). Furthermore, the gold shell enabled dark field imaging to confirm the presence of MCNPs inside the NSCs. This was the very first demonstration to show the utilization of MNPs for the delivery of small molecules into stem cells to induce differentiation.

### 1.3.2 Magnetic Nanoparticles for Stem Cell Labelling and Tracking

The second utilization of MNPs for stem cell-based application is stem cell tracking. The end goal for any stem cell based research is successful transplantation into diseased or injured patients for regeneration. For this, one important criterion is to track the location of the cells after transplantation, ideally in a safe and non-invasive manner. For this purpose, MNPs can be used for stem cell–based therapies to track stem cell migration and localization in vivo because of their unique magnetic properties that enable imaging techniques such as magnetic resonance imaging (MRI). Among the available in vivo
imaging techniques applicable for stem cell monitoring, MRI is particularly promising because it can provide high spatial resolution without compromising the patient’s care. Stem cells, especially, mesenchymal stem cells (MSCs), have been increasingly utilized for \textit{in vivo} transplantation, and it is highly desired to track the location of these transplanted MSC in real-time. For this purpose, MNPs can be loaded into MSCs before transplantation, and their location can be imaged in real-time using MRI imaging. For example, rat MSCs secreting neurothrophic factors were labeled with MNPs and transplanted into Huntington’s disease rat models.\textsuperscript{45} After 18 days, the animals were sacrificed and their brains were imaged to access the migratory path of the transplanted cells. High resolution two dimensional and three dimensional MRI revealed that the transplanted cells migrated along a distant route towards the diseased site (\textbf{Figure 1.4}).

\textbf{Figure 1.4: Tracking Transplanted Stem Cells Using Magnetic Resonance Imaging (MRI) \textit{In Vivo}.} Mesenchymal stem cells (MSCs) were loaded with magnetic nanoparticles (MNPs) and transplanted into rat brains. Throughout the time period, high resolution MRI images revealed that cells migrated along the distant route toward the lesion. The black circle represent the location of the induced lesion. White arrows point to MNP-loaded MSCs.
This confirmed that MSCs can not only seek lesioned regions \textit{in vivo}, but that MNP labeled cells can be tracked via non-invasive MRI imaging even 19 days post-transplantation. Based from this study, it is evident MNPs are an invaluable tool for tracking stem cells.

In another exciting application of MNPs for monitoring stem cell migration \textit{in vivo}, neural stem cells (NSCs) were extracted from patients suffering from traumatic brain injury, and these NSCs were labeled with MNPs. Then, approximately 50,000 of these MNP labeled-NSCs were transplanted into the brain injury site. By utilizing MRI imaging over a 10 week period, the progression and migration of the MNP-labeled NSCs was tracked, and revealed migration from the injection sites to white and gray matter. This phenomenon was not observed in patients received unlabeled cells. Furthermore, it was confirmed that the magnetic signal was indeed from the MNP-labeled NSCs, and not macrophages that engulfed the NSCs, through double fluorescent imaging. It is evident that MNPs are great contrast agents for real-time imaging for stem cell therapies; however, there is one drawback that needs to be addressed. MNP-labeled cells transplanted into the body may not discriminate between labeled dead and live cells. Therefore, if the MNP-labeled cells die after being transplanted, the signal from the MNPs inside the cells will still persist. To test this effect, mice were transplanted with MNP-labeled MSCs and MNP-labeled dead MSCs in the spinal cord, and after 6 weeks, MRI imaging detected a persistent signal from both conditions. This implies that even if cells can be tracked \textit{in vivo} using MNPs and
MRI imaging, an alternative method such as fluorescence imaging has to be utilized to confirm the viability of transplanted cells.

1.3.3 Externally Guiding Transplanted Stem Cells to Target Site In vivo

Even after stem cells are transplanted in vivo, there is no guarantee that they migrate or localize to the area of the diseased or injured site. This limits the full potential of stem cell for regenerative medicine application in vivo. Hence, researchers are exploiting the magnetic properties of MNPs to selectively guide transplanted stem cells to the lesioned site where stem cells are required. For example, in one recent study, MNPs were coated with a polyethelyne glycol in increase biocompatibility and delivered to human MSCs (hMSCs). Then it was confirmed that these MNPs localize in the lysosomes on the hMSCs and are not toxic to cell for a prolonged period. Then they tested the response of MNP-loaded hMSCs under both static and non-static conditions. Under static conditions, the MNP-loaded hMSCs were plated over an array of magnets for 4 hours, which caused the cells to accumulate of the sites with the highest magnetic gradient (Figure 1.5a). Using mathematical models to mimic bloodstream, the response of hMSCs was tested, and revealed that MNPs accumulated to the region of highest magnetic strength (Figure 1.5b). More interestingly, the MNP-loaded hMSCs were injected into the tail vein of mice, with a magnet placed on the proximal portion, which resulted in over a 6 fold increase of accumulation of hMSCs in the tail (Figure 1.5c). This result has immense implications for
In vivo experiments because transplanted cells loaded with MNPs can now be guided to the target site using external, non-invasive methods such as a magnet. Stem cell transplantation for regenerative medicine is a highly promising and pursued field of research, and MNPs provide an effective and non-invasive method to track the progression and precise location in vivo.

Figure 1.5: Guiding In Vivo Localization of MNPs Using External Magnets. A) To demonstrate that MNPs are precisely controlled by the location of a magnetic field, a magnet array with spherical patterns was placed underneath a solution of MNPs, and resulted in MNPs localizing to locations of highest magnetic strength. B) To simulate MNPs flowing in the bloodstream, a MNP solution passed through a tube with a magnet underneath and the localization of the MNP (red) is dictated by the flow rate of the solution. C) MNPs were intravenously injected into the distal portion of the mouse tail vein while a magnet was placed at the injection site. High signal in the tail vein of the mice with the magnet confirms that localization of MNPs can be externally controlled by a magnet.
1.4 SILICA NANOPARTICLES IN STEM CELL BIOLOGY

Silica nanoparticles (SNPs) are a class of nanoparticles comprised of SiO2 and are used extensively for biomedical applications. Their inert properties, small and tunable diameters, and biofunctional capabilities, make SNPs an attractive nanomaterial for biological applications. In fact, in 2011, an investigational new drug application for exploring SNPs for targeted molecular imaging was approved by the US Food and Drug Administration for an in-human clinical trial, thus highlighting SNPs as an effect platform with potential for clinical translation.

SNPs are can be categorized into two major categories: nonporous (solid) SNPs and mesoporous SNPs. Nonporous SNPs are solid, smooth nanoparticles and deliver biomolecule cargo through encapsulation within the SNP or through conjugation on biomolecules on the surface. On the other hand, mesoporous SNPs contain numerous pores (2 – 50 nm in size) on the surface that can hold biomolecule payloads for delivery. The pores are ‘capped’ with a gatekeeper molecule which functions to prevent the release of the payload until the gatekeeper molecule is degraded by intracellular enzymes or opened through external stimuli signals that alter the molecule conformation, thus allowing controlled release of biomolecule payload. The release profile of nonporous SNPs is controlled by the linker molecules or degradation of the silica matrix. Nonporous SNPs can be synthesized in various sizes and the pore size in mesoporous SNPs can be
easily tuned based on the synthetic protocol. Both nonporous and mesoporous SNPs have found their niche in stem cell biology for various applications including stem cell differentiation, stem cell imaging, and \textit{in vivo} real-time stem cell tracking.

1.4.1 Nonporous Silica Nanoparticles for Stem Cell Imaging and Differentiation

Nonporous SNPs have a great multifunctional surface that enables conjugation of active biomolecules for delivery into stem cells.\textsuperscript{53} As a result, SNPs have been demonstrated to deliver differentiation-specific molecules into stem cells for inducing differentiation and conversion into desired lineages. In one demonstration, SNPs were functionalized with insulin and delivered to rat MSCs to induce adipogenic differentiation.\textsuperscript{38} Specifically, researchers first showed a systemic study confirming the biocompatibility of the SNPs with rMSCs that revealed high biocompatibility. Furthermore, high resolution imaging showed that internalization of SNPs by rMSCs had no effect on the cellular structure of organelles. When the SNP-insulin conjugates were delivered rMSCs, successful differentiation into adipogenic tissue was observed, thus demonstrating that the biological activity of insulin was not affected by conjugation to SNP. Hence, SNPs can be established as effective biocompatible carriers of molecules to induce stem cell differentiation.

In addition to their multifunctional surface capable to delivering biomolecules into stem cells, SNPs have a unique intrinsic properties that make them especially attractive for stem cell transplantation applications. SNPs can be detected and visualized using
Ultrasound due to their high impedance mismatch. Ultrasound is a promising tool for stem cell therapy because of its high resolution, low cost, and high depth penetration. Moreover, ultrasound is readily available to clinicians and easy to use, thus making it applicable for stem cell tracking after implantation. One landmark demonstration using ultrasound and SNPs for live stem cell tracking showed the effectiveness of this technique, wherein researchers encapsulated 300 nm SNPs with a fluorescent dye and the element Gadolinium to enhance MRI contrast and ultrasound imaging (Figure 1.6a). These SNPs were then delivered to human MSCs, and imaging showed intracellular aggregation of SNP, which actually enhanced the ultrasound signal without influencing cell behavior or metabolism. The SNP-loaded hMSCs made them applicable for cell sorting through their fluorescence signal. The SNP-loaded hMSCs were then transplanted into mouse model via injections, and after only 11 seconds, researchers were able to use ultrasound imaging to identify the exact location of cells and assess the possibility of a mis-injection (Figure 1.6b). Furthermore, MRI was utilized after the ultrasound-guided delivery of SNP-loaded hMSCs into mouse cardiac tissue. Compared to traditional methods, the SNP loading increased the ultrasound and MRI contrast of labeled hMSCs by over 700% and 200% respectively! Even after 13 days of implantation, the ultrasound signal and MRI contrast could still detect and identify the location of the transplanted hMSCs (Figure 1.6c). Lastly, researchers performed a series of experiments to evaluate the impact of SNPs on hMSCs, and found that all cellular functions including proliferation, cytokine expression, metabolic activity,
were unaffected. Overall, the use of nonporous SNPs for stem cell tracking and stem cell differentiation is a highly promising area of research with potential for translation into the clinic.
1.4.2 Mesoporous Silica Nanoparticles for Delivery of Differentiation-Specific Factors to Stem Cells

The second class of SNPs are mesoporous SNPs, which have pores on the surface in which biomolecules can be embedded and later released in the cell. Taking advantage of this unique feature, researchers demonstrated that differentiation-specific biomolecules can be loaded in the pores to induce stem cell differentiation. Bone morphogenetic proteins (BMPs) are factors that can induce osteogenic bone differentiation. Researchers embedded BMPs into mesoporous SNPs and delivered them into adipose-derived MSCs, with the goal that after the cells uptake the mesoporous SNPs, BMPs would be released from the pores to induce differentiation. Histological staining revealed successful osteogenic differentiation in a highly efficient manner. Comparatively, controls studies with BMPs delivered without mesoporous SNPs showed minimal differentiation.

Generally, stem cells are extremely sensitive to intracellular introduction of foreign inorganic matter, especially embryonic stem cells (ESCs). Just the presence of inorganic matter causes the stimulus of unwanted signaling pathways that can disrupt normal cells function and differentiation capacity. But a research group recently demonstrated that delivery of peptides using mesoporous SNPs does into have any adverse side effects on ESCs, and instead, promotes targeted differentiation. The mesoporous SNPs were loaded with two peptides, Cintrofin and Gliafin. Cintrofin has been shown to induce neuronal
differentiation and promote survival, while Gliafin has been shown promote neurite outgrowth. When these two peptides were loaded into mesoporous and delivered into ESC-derived motor neuron precursor cells, differentiation into motor neurons (MNs) that exhibit neurite branching was observed. Moreover, these induced MNs displayed electrophysiological properties with a resting membrane potential close to physiological range that would be driven to high spiking frequencies. Then, these ESCs were loaded with mesoporous SNPs and transplanted into mice models for *in vivo* differentiation and integration into the mice neural network. After 2 weeks, the condition with loaded mesoporous SNPs showed a neurite outgrowth and the volume of cells was almost 10 times larger than control conditions that lacked and SNP and peptides. After 2 months, the condition with mesoporous SNPs loaded ESCs showed extensive neurite arborizations and expression of prominent markers such as ChAT, while control conditions showed minimal expression. Taken together, these results suggest that co-transplantation of ESCs loaded with mesoporous SNPs can increase transplant size, improve survival, and induce neurite outgrowth.

The development of induced pluripotent stem cells (iPSCs) as a robust source of embryonic-like cells, with the ability to differentiate into almost any cell type, has opened the door for stem cell therapies with potential clinical translation. The first ever demonstration of using SNPs with iPSCs was reported in 2013, wherein researchers evaluated the sensitivity of iPSCs to SNPs and the differentiation capacity of iPSCs
transfected with DNA-loaded SNPs (Figure 1.7a). First, because iPSCs are difficult to transfect and are extremely sensitive to foreign inorganic matter, the uptake dynamics of

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**Figure 1.7: Mesoporous SNPs for Cell Labeling and Differentiating induced Pluripotent Stem Cells (iPSCs).** A) Positively charged mesoporous SNPs were functionalized with a HNF3β-plasmid-DNA (pHNF3β), and delivered to iPSCs. The treated iPSCs showed differentiation capacity and differentiated into hepatocyte-like cells with mature functions within 2 weeks. B) Comparing uptake efficiency of different charged mesoporous SNPs shows that positively charged SNPs have greatest uptake. C) Immunofluorescence analysis for the expression of hepatic markers HNF3β (green) and HNF4α (red) in iPSCs treated with loaded mesoporous SNPs. 1/16 and 1/128 refers to ratios of delivery (scale bar = 200 μm).
mesoporous SNP was evaluated. Three types of mesoporous SNPs were tested, positively charged, negatively charged, and neutral, and the response to iPSCs was carefully observed and resulted in the identifying that positively charged mesoporous SNPs were more efficiently internalized by the iPSCs (Figure 1.7b). Next, to ensure that the intracellular presence of mesoporous SNPs in iPSCs did not impair the function, metabolism and differentiation capacity of iPSCs, various experiment were performed and showed that cell proliferation, pluripotency, and in vivo teratoma formation were not affected. In the final experiment, the mesoporous SNPs were loaded with an HNF3β-plasmid-DNA, which has been shown to induce hepatocyte differentiation. The iPSCs transfected with these mesoporous SNPs loaded with HNF3β exhibited successful differentiation into functional hepatocyte-like cells (Figure 1.7c). These results not only demonstrate the potential for stem cell labeling using mesoporous SNPs, but also that they can act as efficient carriers of differentiation-specific biomolecules into sensitive cells such as iPSCs and induce their differentiation.

1.4.3 Hybrid Silica-Magnetic Nanoparticles Enhance Stem Cell Tracking

SNP and mesoporous SNPs have a unique chemical and synthetic property of being able to integrate with other types of metallic nanoparticles compositions such as gold and iron oxide. These hybrid SNPs are fabricated with a gold shell and a SNP core, or SNPs can act as a shell around magnetic iron oxide nanoparticle cores. The added benefit of
these hybrid SNPs has a synergistic effect in terms of biomolecule loading and contract imaging. A landmark study demonstrated the feasibility of incorporating a magnetic iron oxide nanoparticle inside SNPs for synergistic stem cell labeling. These hybrid SNPs were 50 nm in diameter, and when they are delivered to hMSCs, sufficient MRI imaging is achieved. Furthermore, when these loaded hMSC are transplanted into mouse model, MRI can also detect their presence. Moreover, the differentiation capacity, proliferation, and viability of loaded hMSCs remains unaffected. Hybrid SNPs can have a big impact in translational medicine because of their biological inertness and synergistic modalities.

1.5 QUANTUM DOTS IN STEM CELL BIOLOGY

Quantum Dots (QDs) are a class of multifunctional, fluorescent semiconductor nanoparticles that exhibit quantum mechanical properties of intrinsic emission profiles. QDs vary in size from 2-50 nm, and the emission properties of QDs is directly correlated to either the size or chemical composition (Figure 1.8a). QDs have a broad excitation spectra and a narrow emission spectra. The QDs emit certain wavelengths of fluorescent light, and thus are excellent nanomaterials for non-invasive in vivo imaging and stem cell tracking. Because QDs are excited by a single UV light source and can emit different wavelengths of fluorescent light, they are ideal tools for multiplex imaging. Moreover, QDs are extremely resistant to photobleaching, meaning that they retain emission intensity and brightness, even after long exposure times, and thus are excellent alternative to
traditional molecular dyes to imaging applications. In recent years, the unique and photophysical properties have enabled researchers to broaden the application scope of QDs for stem cell applications because QDs have a multifunctional surface, which, in addition to fluorescent imaging, allows them to simultaneously deliver functional biomolecules. This combinatorial advantage of QDs has propelled them for stem cell applications.

1.5.1 Quantum Dots for Embryonic Stem Cells Labelling

The most prominent stem cells with highest in vivo potential are embryonic stem cells (ESCs) because of their wide differentiation capacity. Therefore, the number of stem cell transplantation studies involving ESCs has been increasing. One important criteria of implantation is to track the location of transplanted ESCs. For this purpose, QDs have emerged as promising probes of stem cell labeling and imaging. But before utilizing QDs for stem cell application, it is critical to evaluate the effect and influence that QDs may have on stem cells, because stem cell are generally sensitive to foreign matter and can alter their metabolism which can adversely impact their differentiation capacity. Furthermore, traditional QDs are synthesized using toxic elements such as a cadmium (Cd) and selenide (Se) based core, which can damage the cell even at low concentrations. However, the use of a ZnS shell prevents the release of the toxic Cd elements, thereby circumventing the cytotoxicity issue.\textsuperscript{64} Therefore, it is important to test the effects of QDs on stem cells. For this purpose, researchers tested the behavior and pluripotent characteristics of ESCs and
kidney stem cells when delivered with QDs.\textsuperscript{65} Results indicate that the native pluripotency markers of ESCs such as Oct4, the proliferation rate, and the viability of ESCs are unchanged by the presence QDs (Figure 1.8b). The differentiation capacity was also not influenced as QD labeled ESCs were able to effectively differentiate to the three germ layers,

Figure 1.8: Fluorescent Quantum Dots (QDs) for Stem Cell Labeling and Tracking. A) QDs of different diameters (top row) and the respective emission wavelength (bottom row in nm), are tunable fluorescent probes. B) QDs incubated in embryonic stem cells (ESCs) show that pluripotent markers such as Oct4 remains unaffected. C) QD-treated ESCs can differentiate into the three germ layers as evidenced by gene expression of each germ layer. D) ESCs treated with QDs differentiate into the germ layers after 4 days. E), F), G) Co-culture of QDs-labeled kidney stem cells (KSCs) with GFP-labeled KSCs (green) confirms that internalized QDs (red) are not transferred to adjacent cells. H) Flow cytometry confirms this result.
endoderm, ectoderm, and mesoderm, with the genetic expression of lineage-specific being identical to differentiated-ESCs lacking QDs (Figure 1.8c, d). Then, to confirm that the labeled-ESCs did not excrete the QDs, which could be potentially be uptaken by adjacent cells, fluorescent imaging revealed the ESCs do not excrete the QDs and that transfer of QDs in co-cultures was minimal (Figure 1.8e-g). Finally, it was determined that even if the ESCs die in culture, the QDs within are also not readily uptaken by adjacent cells. Hence, based on these results, we can deduce that QDs are relatively safe for stem cell applications with minimal side effects.

In another demonstration, researchers sought to evaluate the effect of QDs on ESC behaviors and the feasibility of using QD-labeled ESCs for transplantation studies.\textsuperscript{66} To ensure that the pluripotency properties of ESCs would not affected by the presence of QDs, they delivered the QDs to ESCs and tested pluripotency markers such as Oct4, and found that expression of these markers remained unchanged. Thus, QDs were established as safe agents for further ESC studies. Then, six different types of QDs were delivered to ESCs, and these QD-labeled ESCs were injected into the backs of nude mice (Figure 1.9a). Fluorescent imaging revealed that using a single UV light source, the emission from the six QD-labeled ESCs can be individually detected simultaneously. Finally, to test the prospects of utilized QDs for long-term stem cell tracking, different amounts of QD-labeled ESCs were injected into nude mice and successive imaging for several weeks in performed, which showed that QDs emit a strong and detectable signal even after 14 days post-injection.
(Figure 1.9b, c). This study opened the door for the use of QDs in stem cell research. For example, in another demonstration, bone-derived stem cells (BDSCs) were labeled with QDs and were injected into the retina to repair retinal injury. After transplantation, the location of the injected stem cells could be tracked and using fluorescent imaging.

1.5.2 Non-toxic Alternative QDs for Stem Cell Biology

Combining different types of nanoparticles is an interesting subset of research because it includes the advantages features from both nanoparticles on a single construct. Furthermore, it is desirable to coat QDs with a specific type of nanomaterial to prevent

Figure 1.9: Embryonic stem cells (ESCs) Loaded with QDs can be Simultaneously Imaged. A) ESCs were labeled with six different QDs and injected subcutaneously onto the backs of nude mice. These labeled ESCs could be imaged with good contrast with a single excitation wavelength. B) Different number of QD-labeled ESCs, $10^4$, $10^5$, and $10^6$, were injected into nude mice and signal was quantified and revealed that the signal of ESCs is proportional to number of cells injected. C) To evaluate the clearance properties of labeled ESCs, mice were injected with QD-labeled ESCs and longitudinal imaging revealed that the QD signal can be detected up to 14 days.
leeching of the toxic CdSe core. For this purpose, a recent demonstration showed that QDs can be coated with silica nanoparticles and still retain its fluorescence properties.\textsuperscript{67} A strong intracellular fluorescence signal confirmed that the QDs were effectively uptaken and were able to emit its signal. Moreover, it was determined that a four hour incubation time is the optimal time for maximal uptake of QDs in stem cells.

Another class of recently developed QDs is called graphene quantum dots (GQDs), which are comprised of nanosized graphene sheets and have intrinsic fluorescent properties.\textsuperscript{68} Graphene is a lattice of sp2-carbon sheets that has attracted significant attention due to this unique properties and vast potential for application is almost every field of research. When these graphene sheets are layered together, nanosized GNPs can be created. The most interesting feature of GDPs is their ability to fluoresce under a UV light source. Taking advantage of this, researchers recently developed a facile approach to synthesize large quantities of GQDs that are suitable for stem cell applications.\textsuperscript{69, 70} Specifically, GQDs were used to label various types of cells including neurospheres cells, pancreas progenitor cells, and cardiac progenitor cells, in an effective manner without any cytotoxicity.

Lastly, conventional QDs are comprised of CdSe, which is quite toxic cells, and hence they are capped with an inert ZnS shell to prevent leaching of Cd or Se into the cells. However, it would be highly desirable to replace these toxic element altogether and using inert elements while preserving the fluorescence properties of QDs. To this end, researchers
have developed a unique and facile method to synthesize QDs comprised of nontoxic elements using a sonochemical approach. Specifically, QDs comprised of zinc, indium, silver, and sulfur (ZAIS-QDs) been developed and demonstrated to be non-toxic to stem cells (Figure 1.10a). Moreover, the most attractive feature of ZAIS-QDs is that the emission profile can be tuned based exclusively on the composition of these starting elements (Figure 1.10b). Hence, a large scale library of ZAIS-QDs was readily synthetized and subsequently transfected into human MSCs with high viability, and showed efficient uptake with a strong fluorescent signal (Figure 1.10c, d). Moreover, the ZAIS-QDs were
functionalized with a nucleic acid for simultaneous gene regulation, thus demonstrating the multifaceted properties of QDs.

1.6 NANOPARTICLE ARRAYS FOR STEM CELL BIOLOGY

The ability to use physical cues such as nanotopographical cues, substrate patterns, and ECM geometries, to control stem cell fate is a highly promising area of research. While most techniques discussed thus far have focused on the delivery of nanoparticles, there is another application of nanoparticles involving surface topography. Instead conjugated biomolecules onto nanoparticles for a forward transfection, a recently developed method involves fabricating an array of nanoparticles and then culturing stem cells directly on these nanoparticles. The physical topographical cues and the micro-surface created by the nanoparticles can act to deliver biomolecules and to induce targeted differentiation and behavioral changes of stem cells.11

1.6.1 Nanoparticle Arrays to Deliver Nucleic Acids for Stem Cell Differentiation

In a recent landmark demonstration, researchers utilized a nanoparticles array to deliver hard-to-transfect biomolecules such as small interfering RNA (siRNA), which effectively regulate gene expression, into neural stem cells (NSCs) to induce neuronal differentiation.11 Silica nanoparticles sizes varying from 100-700 nm in diameter, were assembled on a glass substrate using a facile centrifugation method to fabricate the
nanotopography-mediated reverse uptake (NanoRU) platform (Figure 1.11a). Then, siRNA molecules were electrostatically conjugated on NanoRU. When NSCs were seeded on these substrates NSCs readily attached to the surface and extended their axons (Figure 1.11–NanoRU-mediated Delivery of siRNA for Stem Cell Differentiation).

A) Silica nanoparticles are assembled on a film and coated with extracellular matrix (ECM) proteins and nucleic acids (siRNA) to develop the nanotopography-mediated reverse uptake (NanoRU) platform. Neural stem cells (NSCs) cultured on this platform uptake the siRNA, which induces their differentiation into neurons. B) Scanning electron microscopy image of neurons (brown) with extended axons cultured on NanoRU wherein the silica nanoparticles (blue) are visible. C) Depending on the size of the silica nanoparticles on the surface, the uptake rate of siRNA is affected, which is reflected by the difference in GFP knockdown. Nanoparticle with 100 nm diameter showed the highest efficiency. D) Fluorescence images showing the differentiation of NSCs into neurons using the NanoRU platform, and E) the expression of the specific markers, Tuj1 (neuronal) and GFAP (glial cells), were quantified.
To test the efficiency, GFP-labeled NSCs were seeded on NanoRU that was conjugated with siRNA specific for GFP. The results showed a remarkable trend of GFP knockdown as the size of the substrate nanoparticles became smaller, with 100 nm SNPs having the greatest GFP knockdown (Figure 1.11c). Thereafter, siRNA specific for the Sox9 gene, which is responsible for regulating neuronal differentiation, was conjugated on NanoRU and NSCs were seeded on top. The NSCs readily took up the siRNA, and after just 7 days, successful neuronal differentiation was induced (Figure 1.11d, e). The NanoRU platform was then compared to traditional methods of siRNA delivery such as commercially available reagents including Lipofectamine. NanoRU was more efficient and resulted in a significantly higher cell viability. Overall, the researchers concluded that based exclusively on the nanotopography created by the nanoparticle array, nucleic acids could easy be transfected into stem cells to regulate their differentiation.

1.6.2 Nanoparticle Arrays to Regulate Neuronal Behaviours of Stem Cells

In another study, the same authors modified the nanotopography by incorporating a sheet of graphene oxide on the surface of NanoRU to differentiate neural progenitor stem cells (hNPSCs) (Figure 1.12a). Remarkably, just due to the influence of the surface, the axons of the hNPSCs started to align (Figure 1.12b-d). The degree of alignment and efficiency of neuronal differentiation was highest when hNPSCs were seeded on a surface
Figure 1.12: Axonal Alignment on Graphene-Nanoparticle hybrid Substrates. A) Schematic diagram depicting the influence of nanoparticle (NP) monolayers coated with graphene oxide (GO) on the alignment of axons extending from human NSCs and their differentiation into neurons. B), C), D), Aligned growth and extension of axons from differentiated hNSCs, and the compass plots showing the variation in the angle of orientation and length of axons.
containing both SNPs and graphene oxide (Figure 1.12c). Furthermore, the results were reproduced on a flexible polymer, which can potentially be used for in vivo applications (Figure 1.12f). Results show a similar trend of axonal alignment and increase differentiation. Conventional nanoparticles-based methods directly conjugate biomolecules on nanoparticles surfaces for forward delivery, but the method of generating a biocompatible platform to stem cells to grow is promising approach because biomolecules are readily uptaken with a synergistic effect of nanotopographical cues to guide stem cell differentiation.

1.7 SMALL MOLECULE SYNTHETIC TRANSCRIPTION FACTORS

TF are multi-domain proteins that function to regulate gene expression. Specifically, there are two primary structural domains of TF work synergistically to regulate gene. The first is called a DNA binding domain (DBD) which serves to target specific sequences on the DNA, and the second is called the activation domain (AD) which serves to recruits factors such as RNA Poly II to the binding site. Working together, yet functioning independently, these two domains of TFs are responsible for regulating gene expression in all types of eukaryotic cells.

The modular structure of TF proteins gives rise to the possible to developing synthetic counterparts that emulate the function of these individual domains. Small molecules called
synthetic transcription factors (STFs) are a class of small molecules and peptides that function to mimic these functional domains.

**1.7.1 Small Molecule DNA-Binding Domain (DBD) Mimics**

In order to regulate the expression of a gene, the gene sequences on the DNA need to be targeted. This function is fulfilled by the DBD domain on TFs proteins, which are highly specific for target gene sequences. Several synthetic counterparts of DBD have been developed to mimic the gene-targeting ability of TFs. DBD target and bind to predefined sequences on the DNA. Examples of synthetic DBD molecules include oligonucleotides, zinc fingers, and small molecules such as hairpin polyamides.

Oligonucleotides are long strands of DNA with sequences complementary to the target sequence, and recognize and bind to DNA by forming triple helix DNA stands. Oligonucleotides have been used to induce expression of a reporter gene in tissue culture cells, albeit with limited expression potency. Another types of DBD are zinc fingers, which are composed of 30 amino acids stabilized by a zinc ion. Zinc fingers recognize and bind to three base pairs in the target DNA, and multiple zinc fingers can be strung together to recognize larger unique genome sequences. These zinc fingers have been widely employed for gene editing applications in culture, and the most successful zinc finger modules have been those that target GNN triplets. However, the zinc fingers are not separable and their
DNA binding influences by adjacent molecules, hence, they are limited wide use as gene expression modulators.

The most effective type of small molecule DBDs are N-methylpyrrole and N-methylimidazole based hairpin polyamides. Through hydrogen bonding, the pyrrole moiety binds to A-T base pairs and the imidazole moiety binds to G-C base pairs. Hairpin polyamides are capable to binding to the minor groove of targeted DNA sequences with nanomolar binding affinity, and because of this high binding affinity, hairpin polyamides can modify gene expression by competitively inhibit binding of endogenous TFs. Moreover hairpin polyamides are particular attractive as tools for gene expression because their sequence can easily be rearranged to target any gene sequence. Hairpin polyamides have been shown to target gene expression in vitro (nuclear extracts) when linked to an AD molecule. Although hairpin polyamides represent a powerful approach to target gene sequences, poor cell membrane permeability limits its potential for live cell culture gene expression. If the hairpin polyamides can be modified to enhance their membrane permeability and cellular targeting capabilities, they would suitable agents for cellular applications involving gene regulation.

1.7.2 Small Molecule Activation Domain (AD) Mimics

After the DBD binds to the target sequence on the DNA, the other TF component called the activation domain (AD) is required to activate transcription. Specifically, the AD
recruits various intracellular proteins and enzymes including RNA Poly II, the mediator complex, and chromatin remodeling complexes such as SAGA and Swi/Snf, mediators, to the DNA binding site. The assembly of these components functions to initiate transcription the targeted gene. Examples of ADs that have been synthesized thus far include the small molecule named Wrenchnolol, which is a highly efficient in membrane penetration but lacks potent expression, peptides that have been demonstrated transcriptional activity but are prone to degradation will be synthesized and tested, and other small molecules that have high intracellular localization but low transcription efficiency.

One AD in particular has been demonstrated to be effective in inducing transcriptional activity. The transactivation peptide (TAP) was shown to stimulate transcription when conjugated to a complementary DBD molecule. However, the TAP is prone to intracellular degradation, and hence was synthesized in the D-form, and because it is unable to activate transcription of endogenous genes, there is a need to modify the TAP peptide to improve its efficiency.

1.8 CONCLUSIONS AND FUTURE OUTLOOK

Nanoparticles represent a powerful and innovate class of materials for applications in stem cell biology ranging from small molecule delivery, imaging, and in vivo tracking. The field of nanotechnology and nanomedicine has made significant strides over the past decade, and now, several promising approaches are reaching in vivo and clinical therapies.
In order to effectively use nanoparticles for stem cell applications, there are three requirements: high biocompatibility, a multifunctional surface to enable conjugation of biomolecules, and properties to enable non-invasive imaging. To this end, many different types of nanoparticle have been developed for stem cell application including magnetic nanoparticles, silica nanoparticles, quantum dots, and hybrid nanoparticles that integrate multiple nanoparticles together. Each of these nanoparticles have their own unique properties in terms of size, composition, biocompatibility, imaging capabilities, and possible side effects.

A majority of applications utilizing nanoparticle involves stem cell labeling and stem cell tracking in vivo. This is because nanoparticles have excellent intrinsic chemical and physical properties that allow efficient imaging after transplantation without any adverse side effects on cells or patients. Perhaps the greatest feature that enables this their small size, which allows them to remain inside cells without causing cellular damage. Furthermore, there is another scope of application of nanoparticles that involves delivery of nucleic acids and small molecules, which are otherwise difficult to deliver, into stem cells for differentiation. This opens up the door to test almost any type of molecule, regardless if its physical and chemical properties are not suitable for physiological environments. Furthermore, targeted delivery into specific intracellular organelles is achievable by adding targeting peptides on the nanoparticle surface. With all these advantages of nanoparticles,
stem cell biologists and clinicians have the ability to choose the proper nanoparticle for their specific application.

The major obstacle that is currently hindering widespread use of nanoparticle for clinical stem cell use, is the possibility of nanoparticles accumulating in organs and the possible side effects. Even though the nanoparticles may be biocompatible, the effect of accumulating nanoparticles in specific organs after transplanting nanoparticles-loaded stem cells needs to be addressed. With the recent development of hybrid nanoparticles that have a synergistic and combinatorial effect in terms on delivery and imaging, the development of safer and nanoparticles seems promising. The future of utilizing nanoparticles for stem cells-based applications and clinical therapies is very bright. With a greater push for stem cell therapies to treat numerous debilitating disease and injuries, more resources are now geared towards developing translational nanoparticle-based platforms for stem cell medicine.

Finally, even though STFs were first introduced over 15 years and has great potential for stem cell based gene regulation, progress has been limited due the following issues: i) poor solubility at physiological conditions and hence cannot permeate the plasma and nuclear membrane, ii) prone to degradation by proteases, and iii) have a limited ability to mimic natural TFs (e.g. in terms of gene activation). Hence, there is need to modify these STF with a multifunctional nanoparticle that can not only enhance the potency of STFs,
but can also serve as a structural platform for enabling STFs to regulate gene expression
and cellular reprogramming.

1.9 REFERENCES FOR CHAPTER 1


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Chapter 2

NanoScript: A Nanoparticle-based Artificial Transcription Factor for Effective Gene Regulation

Transcription factor (TF) proteins are master regulators of transcriptional activity and gene expression. TF-based gene regulation is a promising approach for many biological applications; however, several limitations hinder the full potential of TFs. Herein, we developed an artificial, nanoparticle-based transcription factor, termed NanoScript, which is designed to mimic the structure and function of TFs. NanoScript was constructed by tethering functional peptides and small molecules called synthetic transcription factors, which mimic the individual TF domains, onto gold nanoparticles. We demonstrate that NanoScript localizes within the nucleus and initiates transcription of a reporter plasmid by over 15-fold. Moreover, NanoScript can effectively transcribe targeted genes on endogenous DNA in a nonviral manner. Because NanoScript is a functional replica of TF proteins and a tunable gene-regulating platform, it has potential for stem cell applications.
2.1 INTRODUCTION

Gene regulation by ectopic expression of key transcription factors (TFs) not only has high impact in the field of biomedical research, but also attracts significant research insight in terms of regulating gene expression. By initiating complex signaling cascades and manipulating genetic circuitry, TFs regulate fundamental cellular behaviors and can also override cellular identity to reprogram and differentiate cells into specific lineages. Transcription factors (TFs) are master regulators of gene expression and are structurally comprised of multiple domains, of which three essential domains are: i) a nuclear localization signal (NLS) domain to shuttle TFs into the nucleus, ii) a DNA binding domain (DBD) which binds to predefined DNA sequences in the promoter region of target genes, and iii) an activation domain (AD) which recruits the transcriptional machinery complex to the binding site to initiate transcription.

Protein therapy, which involves delivering proteins into cells to replace dysfunctional proteins, holds immense potential for applications focusing on regulating gene expression and cellular behaviors such as cellular reprogramming, cancer treatment, and stem cell therapy. Numerous approaches including electroporation, nanocapsules, lipid micelles, polymer-based carriers, and nanoparticle-based delivery, have been developed for intracellular protein delivery. However, practical application of these methods for gene-regulating applications is limited because proteins, especially TFs, which are exogenously
introduced into the cells, have low delivery efficiency, cannot regulate genetic pathways at the transcriptional level, lack cell-specific targeting capabilities, and above all, are extremely vulnerable to degradation by intracellular proteases.\textsuperscript{10,11}

Rather than designing just another protein delivery vehicle, we sought to develop an innovative platform that replicates the fundamental function of TF proteins using a nanomaterial-based small molecule approach. Nanomaterials, such as gold nanoparticles (AuNPs), have desirable physiochemical as well as nano-structural properties, and have been successfully established for biological applications.\textsuperscript{11} Along with these unique attributes, the biological inertness and high stability in physiological conditions make AuNPs excellent carriers of small molecules and biomolecules. Recently, chemical biologists developed a class of small molecules, called synthetic transcription factors (STFs), which have been demonstrated to mimic the function of each TF domain, albeit with limited success.

Herein, we demonstrate development of a novel nanoparticle-based artificial transcription factor, termed \textbf{NanoScript}, which replicates the multi-domain structure and gene-regulating function of natural TFs (\textbf{Figure 2.1a}). NanoScript replicates the multi-domain structure of TF proteins because the three major components, which represent the three major domains (NLS, DBD, and AD) found on endogenous TFs, are tethered together in close proximity on a single AuNP (\textbf{Figure 2.1b}). In addition to serving as a
delivery vehicle for STFs, the AuNP itself serves as a functional component of the NanoScript because it mimics the linker domain (LD) of natural TF proteins (Figure 2.1b). NanoScript emulates the gene-regulating function of TFs because the three STF components, which include i) a NLS peptide ii) a hairpin polyamide DBD, and iii) a transactivation peptide AD, all function synergistically to regulate transcriptional activity of targeted genes in a non-viral manner (Figure 2.1c).
The NanoScript platform presented here has several advantageous features such as a multifunctional AuNP surface that allows attachment of all STF components with a flexible density onto a single nanoparticle, a compact hydrodynamic size (<42 nm diameter) for effective nuclear localization and DNA intercalation with intrinsic multivalent interactions, an ability to target selective predetermined genes, a non-integrative mechanism for regulating genes. As a result, our carefully designed NanoScript can localize within the nucleus and effectively initiate transcriptional activity of both a reporter plasmid and endogenous genes. It is important to note that NanoScript does not deliver TF proteins, but instead, acts as a functional platform that is designed to behave like them.

2.2 METHODS

**Synthesis of Synthetic Transcription Factors (STFs):** The transactivation peptide, having a sequence of CGSDLDDFDDMLGDSDLDDFDDMLGDMLGS-NH₂, was purchased from Invitrogen. The nuclear localization peptide, having a sequence of CGGPKKKKRKVED-OH, was purchased from GenScript. All peptides were stored and dissolved as per the manufacturer recommendations.

All machine-assisted Py-Im polyamide synthesis was performed on 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. Reaction steps in the synthetic cycle were as follows: i) deblocking steps for 4 min x 2, 20% piperidine in DMF; ii) coupling step for 60 min, corresponding carboxylic
acids, 1H-Benzotriazolium, 5-[bis(dimethylamino)methylene]-2chloro-hexafluoro phosphate (1-),3-oxide (HCTU) (88 mg), diisopropylethylamine (DIEA) (36 µL); iii) washing steps for 1 min x 5, DMF. Each corresponding carboxylic acids in coupling step were prepared in 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. A dried resin was cleaved with 0.4 ml of 3,3’-diamino-N-methyldipropylamine for 3 h at 55 ºC. The reaction mixture was filtered, trituted from CH2Cl2 and Et2O, to yield crude as yellow powder. The crude was purified 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 under 254 nm) to yield Py-Im polyamides as white powder.

(a) Analytical HPLC: Retention Time = 14.7 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) δ 10.23 (s, 1H; NH), 9.97 (s, 1H; NH), 9.94 (s, 1H; NH), 9.93 (s, 1H; NH), 9.90-9.89 (t, J = 4.2 Hz, 3H; NH), 9.84 (s, 1H; NH), 8.10-8.04 (m, 3H; NH), 7.43 (s, 1H; CH), 7.28-7.21 (m, 3H; CH), 7.18-7.16 (m, 4H; CH), 7.07 (m, 3H; CH), 6.91-6.87 (s, 4H; CH), 3.95 (s, 3H; NCH3), 3.86 (s, 3H; NCH3), 3.85 (s, 9H; NCH3), 3.84 (s, 3H; NCH3), 3.83 (s, 3H; NCH3), 3.81 (m, 3H; NCH3), 3.80 (m, 3H; NCH3), 2.74 (d, J = 4.2 Hz, 6H; CH2), 2.37-2.27 (m, 6H; CH2), 2.03 (s, 3H; COCH3), 1.91-1.90 (m, 4H; CH2),
1.81-1.76 (m, 6H; CH2). ESI-TOF-MS (positive) m/z calcd for C63H81N22O11+ [M+H]+ 
1321.65; found 1321.64.

**SPR Binding Affinity:** The Surface Plasmon Resonance (SPR) assays were performed using a BIACORE X instrument. Biotinylated hairpin DNAs were purchased from JBioS (Tokyo, Japan) and the sequences are shown in Tables. Hairpin biotinylated DNA are 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, 3mM 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 µl/min. To measure the rates of association (ka), dissociation (kd) 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 to give better fitting. The closeness of fit is described by the statistical value: \( \chi^2 = \frac{\sum (r_f - r_x)^2}{n-p} \) (\( r_f \) = fitted value at a given point, \( r_x \) = experimental value at the same point, \( n \) = number of data points, \( p \) = number of fitted parameters).
**Synthesis of Gold Nanoparticles:** The gold nanoparticles with an approximate diameter of 9 nm were prepared by the Ferns method of citrate reduction of HAuCl₄ following established protocols. All glassware was cleaned in aqua regia (3 HCl: 1 HNO₃, handle with extreme caution!), then rinsed with nanopure water and oven dried. A 50 mL aqueous solution of 1 mM HAuCl₄ was heated to a reflux while stirring. Then 8 mL of 1% (by weight) sodium citrate was quickly added, resulting in a change in solution color from yellow to ruby red. After the color change, the solution was heated to reflux for another 5 minutes, then cooled to room temperature and filtered using a 0.45 μm syringe filter. Size distribution was characterized using transmission electron microscopy (TEM) and dynamic light scattering (DLS), and concentration was obtained using UV-vis spectroscopy.

**Construction of NanoScript:** 10 nm gold nanoparticles (AuNPs) were conjugated to ligands using a two step method. The first step involves ligand exchange on the AuNP with the linker molecule 11-mercaptoundecanoic acid (MUA). First, from a stock solution of MUA dissolved in ethanol, 1 mM MUA was added to the AuNP solution (pH=11, NaOH) and allowed to stir at room temperature for 24 hr. The solution was then filtered three times using a 10,000 MWCO filter (Millipore) and re-suspended in distilled water. The second step involves conjugating the ligands to the AuNP via the carboxylic acid of the MUA. To the AuNP solution, adjusted to pH=6.5 by adding 5 mM MES (Hampton Research), was added 0.3 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)
(Sigma) and 0.75mM of N-hydroxysuccinimide (NHS) (Acros Organics) and allowed to stir for 2 hr at room temperature. The solution was filtered three times using a 10K MWCOF, and re-suspended in 1mM MES water. Immediately afterwards, a solution containing 10 molar excess of nuclear localization signal (NLS) peptide, trans-activation peptide (TAP), and hairpin-polyamide DNA Binding Domain (DBD) with a mole ratio of 7:2:1 (NLS:TAP:DBD) was added drop-wise to the AuNP solution, adjusted to pH=7.2 by adding 50 mM HEPES (Cellgro) prior to addition of biomolecules, and allowed to stir for 2 hr. The AuNP solution was filtered three times using a 10,000 MWCO to remove unreacted molecules.

The dye-labeled NanoScript, used for tracking intracellular localization, was constructed by modifying the AD (the transactivation peptide) with a fluorescent dye (Alexa Fluor 568 Hydrazide, Invitrogen). Specifically, the dye was conjugated to the transactivation peptide (TAP) via EDC/NHS coupling as described above.

**NanoScript Characterization:** The NanoScript construct was characterized using multiple techniques. First, the concentration of the gold nanoparticles and confirmation of conjugation was found using UV-visible absorption spectra (Varian Cary 5000 UV Vis-NIR Spectrophotometer). Second, Dynamic Light Scattering (Malvern Zetasizer Nano-ZS90) was utilized to measure the size and zeta potential (surface charge) of the AuNP construct after each conjugation step to find the hydrodynamic diameter and surface
charge respectively. Third, the morphology of the AuNP core was determined using transmission electron microscopy (TEM) analysis. The AuNPs were drop-cast on the Holey-carbon grids (Electron Microscopy Sciences), allowed to dry overnight under vacuum, and subsequently imaged using a JEOL JEM-2010F high-resolution TEM operated at an accelerating voltage of 200 kV. Finally, the amount of peptides on NanoScript’s surface was calculated by performing HPLC (Agilent LC 1100) using a Zorbax Extend-C18 Solvent Saver Plus, 3.5 μm, 3.0 × 150 mm column. The peptide solution before conjugation and the supernatant solution after conjugation, which contained unreacted peptides, were both analyzed using HPLC. The exact number of moles for each peptide was calculated using a standard curve comparing the concentration and area. The difference in moles for each peptide before and after conjugation was calculated. The ratio of the difference in moles is attributed to the ratio on the nanoparticle. For this experiment, after the nanoparticles were centrifuged at 12,000 RPM for 20 minutes, the supernatant was collected and analyzed.

**Cell Transmission Electron Microscopy (TEM):** HeLa cells were cultured with NanoScripts using the same method as above, but in a 6 cm cell culture dish. 48 hr post-transfection, the cells were trypsinized and fixed with Trump’s Fixative (Electron Microscopy Sciences) for 1 hr, washed with sodium cacodylate buffer (Electron Microscopy Sciences), suspended in a 1% osmium tetroxide solution for 1 hr, washed with water, and then progressively dehydrated with ethanol (50, 70, 80, 95, 100%). Then the cells were embedded in epoxy resin using the Low Viscosity Embedding Media Spurr's Kit
(Electron Microscopy Sciences) following the manufacturer’s protocol. The images were obtained with the JEOL 100CX TEM.

**Reporter Plasmid Design:** The reporter plasmid was derived from the pSEAP2-Basic plasmid (Clontech), which lacks a eukaryotic promoter and enhancer sequences. Specifically, the STFM response element, which contains 6 sequences recognized by the DBD was cloned into the pSEAP2-Basic plasmid using the KpnI and HindIII restriction sites. The sequence of this STFM response element can be found in Figure 2.2. The sequence of the plasmid was confirmed by sequencing and restriction enzyme analysis.

**HeLa Cell Culture and NanoScript Uptake:** HeLa cells were cultured in Dulbecco’s Modified Eagle medium with high glucose (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, 1% glutamax (Invitrogen) and 1% streptomycin-penicillin antibiotic in a 37°C humidified incubator with 5% CO2. Prior to transfection, 25,000 HeLa cells were seeded in a well of 24 well-plate. After 24 hr, the cells were incubated with a mixture of the 1 nM NanoScript solution, 0.5μg of the reporter plasmid, and X-tremeGENE (Roche) in Opti-MEM medium (Invitrogen). After 4 hr, the cells were washed twice with PBS and fresh culture medium was added. After 48 hr, the alkaline phosphatase level in the culture medium was tested using the SEAP Chemiluminescence Kit 2.0 (Clontech) following the manufacturer protocol. An MTS assay (Promega) was also performed at this time to test cell viability.
**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).

Primers sequences are in the table below.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer (5’ – 3’)</th>
<th>Reverse Primer (5’ – 3’)</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGALS8</td>
<td>CCTATGACACGCTTTTCCAAAAGA</td>
<td>CAGCACCATAATCAGATCTCAA</td>
<td>56</td>
</tr>
<tr>
<td>PPAP2A</td>
<td>GGCAGGGTGTCTCTTCTTATTAG</td>
<td>CAGTGTGGGGCGTAAAGGT</td>
<td>120</td>
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<tr>
<td>BAG4</td>
<td>ACTTACCGTTTCTCTGGCAAC</td>
<td>GGGTGCTTCAGTCTGACAGT</td>
<td>81</td>
</tr>
<tr>
<td>EGLN3</td>
<td>TCCTGCGGATATTTCAGAGG</td>
<td>GTTCCTACGATCTGACCAGAA</td>
<td>93</td>
</tr>
</tbody>
</table>

**Immunocytochemistry:** To investigate the nuclear localization of the dye-labeled (Alexa Flour 568, Life Technologies) NanoScript in HeLa cells 48 hr post-transfection, the media was removed and the cells were fixed for 15 minutes in formalin (Sigma) followed by two washes with PBS. The nucleus was stained with DAPI (Life Technologies) for 30 minutes and then washed with PBS three times. Slides were imaged on an OMX microscope in widefield (non-SIM) mode. A 405nm / 200mW laser was used to excite DNA stained with DAPI, and a 593nm / 500mW laser was used to excite the Texas Red dye. 200msec exposures were used. Z stacks were taken from 8 to 10µm depending on cell Z depth, at 0.125µm section thickness. Emission light was collected with a 100x, 1.4NA oil immersion objective (Olympus) and emission channels were directed onto one of two separate Photometrics CascadeII:512 EMCCD cameras, with a final lateral pixel size of 0.0792µm. Constrained iterative deconvolution was used with a measured point spread function to de-blur images. Images from separate cameras were aligned in XY using a
transformation matrix derived from previously recorded images of 100nm fluorescent latex beads, and in Z using the DAPI image recorded onto both cameras.

**Sample Preparation and ICP-OES Measurement:** After HeLa were seeded in a 24-well plate (20,000 cells/well), 1 nM of NanoScript was added; and after 4 hours, the cells were washed with PBS three times and detached using trypsin. After centrifugation, the supernatant was removed, and the cells were resuspended in PBS and counted using a hemocytometer. After centrifugation, the supernatant was removed and 25 µL of lysis buffer was added to the cell pellet. Then 250 µL of aqua regia (caution: extremely reactive) was added and allowed to sit overnight in an uncapped eppendorf tube. The next day, the solution was transferred to 9.725 mL 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 established conditions. A standard curve comparing the number of gold nanoparticles to the Au intensity was established using known nanoparticle concentrations.

### 2.3 RESULTS AND DISCUSSION

#### 2.3.1 Construction and Characterization of NanoScript

NanoScript was synthesized by assembling three essential components on AuNPs. The first is a hairpin polyamide structure (representing the DBD) which was specifically chosen because of its high specificity to target DNA sequences and exceptionally high binding
affinity that is comparable to naturally occurring DNA-binding proteins. The hairpin polyamide is comprised of N-methylpyrrole (Py) and N-Methylimidazole (Im) amino acids that bind to complementary A-T and G-C motifs on the DNA respectively. Using solid-phase synthesis, a polyamide was synthesized with amino acids arranged in the order of

![Image](image_url)

Figure 2.2: The Py-Im Hairpin Polyamide has High Binding Affinity for the Target Sequence. (a) The hairpin polyamide structure of the DBD containing pyrrole (Py) and imidazole (Im) motifs, that bind to A-T and G-C base pairs respectively, are arranged to target the 3’-WWWWGW-5’ sequence (W=A or T). The hairpin polyamide DBD is anchored with a dimethylaminopropylamine (DAMPA) moiety to enable conjugation. (b) SPR sensorgrams show the interaction of varying polyamide DBD concentrations with complementary hairpin DNAs. The equilibrium constant (KD), which is indicative of the binding affinity, was determined by the ratio of the dissociation constant (kd) to the association constant (ka).
ImPyPyPy-γ-PyPyPyPy-β-Dp-NH₂ (γ is γ-aminobutyric acid, β is β-alanine, and Dp is dimethylaminopropylamide), to recognize the 5’-WGWWWW-3’ (W=A or T) DNA sequence (Figure 2.2a). This sequence was specifically chosen to recognize the matching DNA sequence of our proof-of-concept reporter plasmid, which will be used to evaluate its gene-regulating functionality (details described below). Using surface plasmon resonance, the binding affinity of the polyamide to its target sequence was determined to have a remarkably high nanomolar affinity with an equilibrium constant of $1.6 \times 10^{-9}$ M (Figure

![Figure 2.3: Binding Affinity of Polyamide to Mismatch DNA Sequence. SPR sensorgrams show the interaction of varying polyamide DBD concentrations with complementary hairpin DNAs. 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 ($k_a$). The mismatched G-C base pair is highlighted in red. Comparing the binding affinity of the polyamide to the target sequence (from Figure 2.2) versus the mismatch sequence, there is a 70 fold difference.](image)

<table>
<thead>
<tr>
<th>$k_a$ (M⁻¹s⁻¹)</th>
<th>$k_d$ (s⁻¹)</th>
<th>$K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1.54 \times 10^4$</td>
<td>$1.76 \times 10^{-3}$</td>
<td>$1.14 \times 10^{-7}$</td>
</tr>
</tbody>
</table>
2.2b). In addition, the polyamide binding affinity to a mismatched DNA sequences decreased by over 70 fold, thus implying the polyamide is specific for its target sequence (Figure 2.3).

The second component (representing the AD) is a synthetic transactivation peptide that was synthesized in the D-form to resist intracellular degradation and is capable of activating transcriptional activity by recruiting mediators, RNA polymerase II, SAGA, and other proteins to the binding site. The third component (representing the NLS domain)

Figure 2.4: Construction and Characterization of NanoScript. (a) 10nm AuNPs are first coated with mercaptoundecanoic acid (MUA), then activated with EDC/NHS coupling chemistry, and finally the small molecules components are assembled to construct NanoScript. This reaction was carried out in a controlled buffered (pH = 6.0-7.4) solution to ensure conjugation through the primary amine, but there is a possibility that NLS binds either directly to the AuNP or through lysine side chains, which should not influence its functionality. (b) The hydrodynamic diameter of NanoScript increases stepwise with the conjugation of functional components. The surface charge of NanoScript also increases stepwise with the addition of more negatively charged components. (c) A TEM micrograph showing a monodisperse and uniform distribution of NanoScript.
is a nuclear localization signal (NLS) peptide derived from the SV40 large T-antigen and functions to shuttle NanoScript into the nucleus.

These three components were then assembled onto a single nanoparticle using a carefully designed conjugation chemistry to develop NanoScript. The NanoScript platform was constructed by first coating AuNPs with mercaptoundeconic acid (MUA) and then conjugating the three STF components via EDC/NHS coupling (Figure 2.4). This reaction was carried out in a controlled buffered (pH = 6.0-7.4) solution to ensure conjugation through the primary amine, but there is a possibility that NLS binds either directly to the AuNP or through lysine side chains, which should not influence its functionality. Adsorption of the STF components on the AuNPs was confirmed by UV spectroscopy,

![Figure 2.5: UV Absorbance of NanoScript Conjugates. Unmodified, citrate-stabilized 10nm AuNPs shows a characteristic peak at 518 nm. After functionalization with MUA and conjugation of STF components, the successive shift in the absorbance peak implies attachment of biomolecules on the AuNP surface.](image)
which indicated a successive shift of the surface plasmon peak (Figure 2.5). Based on previous reports, we are confident the DBD conjugated to NanoScript binds preferentially to the target sequence. In terms of the STF ratio on NanoScript, we speculated a high ratio of NLS would be required because translocation of NanoScript into the nucleus is one of the most critical barriers for effective gene activation. Furthermore, we anticipated that a minimal ratio of the polyamide DBD would be required because of its exceptionally high binding affinity to DNA and that the ratio of AD be doubled in order to mimic the potent endogenous TF p53. Therefore, we designed NanoScript to have an optimum ratio of STF components and quantified the surface ratio using high pressure liquid chromatography (HPLC) analysis (Figure 2.6).

The physical properties were characterized using dynamic light scattering, which determined the hydrodynamic diameter of NanoScript to be 34.0 ± 2.3 nm and the surface charge to be -32.5 mV (Figure 2.4b). This hydrodynamic diameter is in excellent agreement to the theoretical value, because based on calculating bond lengths, we predicted

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Mole Ratio of STFs on NanoScript Surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLS</td>
<td>68.2 ± 1.0%</td>
</tr>
<tr>
<td>TAP</td>
<td>22.8 ± 2.6%</td>
</tr>
<tr>
<td>DBD</td>
<td>9.0 ± 2.1%</td>
</tr>
</tbody>
</table>

Figure 2.6: Ligand Ratio on NanoScript. HPLC analysis was performed to calculate the moles of each peptide in both the solution before conjugation and the supernatant solution after conjugation containing unreacted peptides. The mole ratio of the difference is the ratio on the peptide on NanoScript.
the theoretical diameter of NanoScript to be 35.2 nm. Electron micrographs of NanoScript confirmed that surface functionalization with STF components did not affect the size distribution or monodispersity of the nanoparticles (Figure 2.4c). The stability of both NanoScript and MUA-coated AuNPs (Au-MUA) was tested in various physiological environments including water, PBS and culture media, and showed that the nanoparticles

![NanoScript Stability](image)

**Figure 2.7: NanoScript Stability.** The MUA-modified nanoparticles and NanoScript were maintained at 37°C in various solutions including: water pH=5.5, PBS pH=7.4, and Cell Culture Media. Over the course of three days, the stability of the nanoparticles was tested using UV-Vis, which revealed only a slight shift in the peak and indicated that the nanoparticles remained stable in these solutions.
remained stable in these environments as indicated by minimal shifts in the absorbance peak (Figure 2.7).

2.3.2 Nuclear Localization of NanoScript

Efficient nuclear localization of NanoScript is especially important because transcriptional activity occurs exclusively in the nucleus. To this end, NanoScript was specifically designed with a small hydrodynamic diameter (~34.0 nm as mentioned above) because the nuclear pore, which tightly regulates cargo transport across the nuclear envelope, has a maximum diameter of approximately 44 nm. The plasma membrane permeability of NanoScript was assessed by first incubating NanoScript with HeLa cells for 4 hours and then quantifying the number of particles that were able to enter the cells using inductively coupled plasma atomic emission spectroscopy (ICP-OES). Analysis revealed that NanoScript was able to efficiently penetrate the plasma membrane in just 4 hours of incubation, as compared to the control (NanoScript without NLS peptide) which showed minimal uptake (Figure 2.8a), which indicates that the NLS peptide also plays a significant role in enabling NanoScript to enter the cell. To monitor intracellular localization, NanoScript was labeled with a fluorescent dye and incubated in HeLa cell cultures. Fluorescence images, taken with a 3D Structured Illumination Microscope, shows that NanoScript is able to target and penetrate the nuclear envelope (Figure 2.8b, Figure 2.9). Additionally, a side-view image indicates that NanoScript is evenly dispersed throughout
the nucleus in the vertical plane, thus confirming that NanoScript is not merely resting on the surface of the nucleus (Figure 2.8c).
Given the potential for degradation of NanoScript components by intracellular proteases, we wanted to ensure that the observed fluorescence overlap was not due to components being cleaved from NanoScript and diffusing into the nucleus, but rather, that the nanoparticles were able to enter the nucleus intact. For this purpose, we performed TEM on cellular cross-sections to determine if intact NanoScript was able to enter the nucleus. The TEM image shows the interface between the cytoplasm and the nucleus, with NanoScript clearly located inside the nucleus (Figure 2.8d, Figure 2.10). While this indicates that the NLS peptide may be initially conjugated on NanoScript, there is high

Figure 2.9: Nuclear Localization of NanoScript. HeLa cells were transfected with NanoScript and imaged 48 hr later. The fluorescence image shows the overlap of NanoScript (red) with the nucleus (blue). (Scale bar = 20 μm)
probability that cytoplasmic proteases degrade the NLS peptide over a longer period of time. Combining the results of the fluorescence and TEM images, NanoScript was demonstrated to effectively enter the nucleus while remaining intact, which would be critical for achieving high transcriptional activity.

**Figure 2.10: Cell TEM of Whole Cell.** A cross-sectional TEM micrograph showing a HeLa 48 hr post-transfection reveals that NanoScript (indicated with light blue arrows) is localized in the cytoplasm, near the outside of the nuclear membrane, and within the nucleus (purple outline).
2.3.3 Transcriptional Activation using NanoScript

The primary function of TFs is to regulate transcriptional activity; hence we evaluated NanoScript’s ability to control transcription and gene expression. As a proof-of-concept experiment, we constructed a reporter plasmid containing a response element with *six tandem copies* of the 5’-*TGTTAT*-3’ sequence, which is selectively recognized by the polyamide DBD molecule ([Figure 2.11, Figure 2.12a]). Transcription of this reporter plasmid produces alkaline phosphatase (ALP), which can be quantified using a colorimetric assay. Since the secreted ALP is directly proportional to the magnitude of induced transcriptional activity, the functionality and potency of NanoScript can be evaluated and quantified. After co-transfecting NanoScript and the reporter plasmid into HeLa cells, ALP levels were measured after 48 hours ([Figure 2.12b]). Analysis revealed that NanoScript induces transcription and overexpresses the reporter plasmid by 15-fold in comparison to unmodified AuNPs ([Figure 2.12c]), while maintaining high cell viability.

![Figure 2.11: Reporter Plasmid Response Element](image)

Response element containing six copies of the targeted sequence (TGTTAT) that secrets alkaline phosphatase upon transcription is located 36 base pairs upstream from the TATA box. This response element was inserted in the pSEAP2-Basic Vector (Clontech), between KpnI (GGTACC) and HindIII (AAGCTT) sites.
Figure 2.12: NanoScript Transcribes a Reporter Plasmid and Activates Endogenous Genes.

(a) The reporter gene containing 6 copies of the response element is located upstream from the TATA Box and secretes alkaline phosphatase (ALP) upon transcription. (b) Schematic representation of co-delivering the reporter plasmid and NanoScript. After NanoScript and the reporter plasmid both localize within the nucleus, transcriptional activity is initiated to produce ALP, which is secreted into the culture media. (c) The ALP fold induction initiated by NanoScript (1 nM) compared to controls which lack individual components (d) Concentration-dependent induction of ALP by NanoScript. (e) HeLa cells were incubated with NanoScript (1 nM) and 48 hours later, qPCR analysis reveals activation of targeted genes. Standard error, mean, and t-test analysis for all experiments was derived from three individual trials. ALP was measured 48 hr post-transfection and fold increase is relative to unmodified AuNP controls. T-test analysis * for $P<0.01$. 
(Figure 2.13). Furthermore, the contribution of each component on NanoScript for initiating transcriptional activity was tested by selectively removing each component. Subsequent ALP analysis of control experiments showed limited gene expression, thus confirming the importance and synergistic activity of each STF component on NanoScript (Figure 2.12c). A positive control included transfecting the same concentrations of DBD and AD molecules as on the nanoparticle to the culture media and showed a 2.2 fold increase in gene transcription, a result which is similar to the previously reported studies. Moreover, when cells were exposed to varying NanoScript concentrations, there was dose-dependent transcriptional activation (Figure 2.12d).

![Figure 2.13: Cellular Viability of NanoScript-treated Cells.](image)

Cell Viability (% of Control) vs. NanoScript Concentration

- **Unmodified AuNPs**
- **NanoScript (0.25 nM)**
- **NanoScript (0.5 nM)**
- **NanoScript (1 nM)**
- **NanoScript (2 nM)**
- **NanoScript w/o NLS (1 nM)**
- **NanoScript w/o AD (1 nM)**
- **NanoScript w/o DBD (1 nM)**

**Figure 2.13: Cellular Viability of NanoScript-treated Cells.** Cellular viability was calculated by analyzing MTS assay activity 48 hr post-transfection. The graph shows the cell viability of various NanoScript concentrations as well as NanoScript controls that lack individual components (w/o = without). Percentages are compared to untreated cells and standard error is calculated from three independent trials.
2.3.4 NanoScript Activates Endogenous Genes

In order to fully emulate the gene-regulating function of TFs and to establish the NanoScript platform suitable for potential biological applications, it is essential that NanoScript can activate endogenous genes on native DNA. For this purpose, several genes including LGALS8 and EGLN3 were identified to contain numerous response elements that complement the polyamide DBD (Figure 2.14). The number of complementary binding sites on endogenous DNA is not expected to be directly proportional to the level of gene activation, primarily due to the nucleosome/histone packaging of DNA and variability in binding site availability. Quantification of endogenous gene activation showed that the expression of LGALS8 and EGLN3 was increased 65% and 58% respectively, relative to unmodified AuNPs (Figure 2.11e). Successful gene activation indicates that NanoScript can interact with endogenous DNA, seek complementary gene motifs, and initiate transcriptional activity of targeted endogenous genes. It is important to

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of Binding Sites on Promoter Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGALS8</td>
<td>36</td>
</tr>
<tr>
<td>PPAP2A</td>
<td>22</td>
</tr>
<tr>
<td>BAG4</td>
<td>11</td>
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<tr>
<td>EGLN3</td>
<td>28</td>
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</table>

Figure 2.14: Consensus Promoter Sequences on Native DNA. Number of binding sites that the polyamide DBD can bind on the promoter sequence of the four overexpressed endogenous genes. Binding sites are within 1000 basepairs 5’ from the transcription start site.
note that the gene activation of the reporter plasmid was expected to be higher than the activation of endogenous genes because endogenous genes are tightly coiled into nucleosomes while reporter plasmids are not, thus making the reporter plasmid more prone to being transcribed. The overexpression of endogenous genes highlights the versatility of NanoScript, thus proving that this platform can behave and function like natural TFs.

2.4 CONCLUSIONS

Most previously reported methods to regulate gene expression involve either viral vectors (e.g. DNA plasmids and retroviruses),33-35 small molecules (e.g. RNAi and synthetic molecules) that regulate translation or target a specific signaling pathway, or nanomaterial-based exogenous cues (e.g. nanotopographical surface patterns). However, NanoScript is the first nanomaterial-based platform that mimics natural TFs and interacts with endogenous DNA to regulate gene expression at the transcriptional level in a non-viral manner.

Several remarkable features of NanoScript enable translation of this platform for applications that require gene manipulation. First, because gene expression by NanoScript is non-viral, this platform is an attractive alternative to conventional viral-based methods for regulating forced gene activation. Second, the multifunctional AuNP surface of NanoScript can potentially allow for attachment of additional moieties such as histone
modification molecules, cell-specific targeting peptides, and RNAi molecules for an enhanced and synergistic regulation of gene expression. Third, the AuNP backbone of NanoScript can be replaced with other types of nanoparticles, such as upconverting nanoparticles and magnetic core-shell nanoparticles, for potential applications such as tracking gene regulation in real-time through infrared imaging or magnetic resonance imaging (MRI) respectively. Finally, the most advantageous feature of NanoScript is its tunable components, wherein, by simply redesigning the hairpin polyamide DBD sequence to target regulatory genes such as those involved in differentiation. However, it should be noted that, due to the short DBD targeting sequence, there is a possibility for interactions with off-target genes; hence, further optimization by designing DBDs with longer sequences to target longer DNA sequences would further enhance the NanoScript platform. Overall, NanoScript platform has great potential to be utilized for stem cell biology or reprogramming applications.

In summary, we have developed an innovative NanoScript platform, which effectively replicates the multi-domain structure and gene-regulating function of naturally occurring TFs. As a proof-of-concept experiment, we show NanoScript to effectively regulate gene expression of both a reporter plasmid and endogenous genes on native DNA. Due to the unique tunable properties of NanoScript, we are highly confident this platform will not only serve as a desirable alternative to conventional gene-regulating methods, but also has
direct employment for applications involving gene manipulation such as stem cell
differentiation, cancer therapy, and cellular reprogramming.

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Chapter 3

Inducing Stem Cell Myogenesis Using NanoScript

This chapter describes 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.
3.1 INTRODUCTION

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.\(^1\) 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 multi-domain structure of TFs is comprised 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 \textit{MyoD}, \textit{Myogenin}, \textit{Myf5}, and \textit{Mrf4}, and have been demonstrated to play a critical role in generating muscle cells from both somatic and stem cells.\(^5,6\) As such, the modulation of MRFs hold tremendous potential to treat incurable degenerative muscle disorders, such as muscle dystrophy.\(^6\)
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),\(^6\textsuperscript{,}^7\) or ii) stimulate production of gene-specific TFs within the cells (using viral vectors and small molecules).\(^9\textsuperscript{,}^10\) 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.\(^10\) Moreover, direct delivery of TF proteins into cells face limitations including intracellular degradation and low delivery efficiency, which leads to limited gene expression.\(^11\) Hence, there is an urgent need to develop an alternative approach that not only overcomes the limitation of conventional methods, but is capable of regulating gene expression and inducing stem cell differentiation in an effective and non-integrative manner.\(^11\)

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.\(^11\) 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 non-viral 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.

We demonstrate that NanoScript can be designed to replicate natural TFs that are specific for myogenic regulatory factors (MRFs) (Figure 3.1). 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 non-integrative approach to successfully differentiate adipose-derived mesenchymal stem cells (ADMSCs) into muscle cells. The gene expression levels induced by NanoScript-MRF is comparable to and even exceeds 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. 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.
Figure 3.1: Schematic Representation of NanoScript-MRF Emulating Transcription Factor Proteins. (a) The 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 gold nanoparticles (AuNPs), the NanoScript platform is fabricated. (b) The myogenic regulatory factors (MRFs), 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 (ADMSCs) induces activation of MRFs, which guides their differentiation into muscle cells.
3.2 METHODS

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

The synthesis of the polyamide was performed as previously reported. 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]-5-chloro-hexafluorophosphate (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 C₉₂H₁₁₅N₃₃O₁₇₂⁺ [M+2H]⁺ 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 HAuCl₄ 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 HAuCl₄ 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.

**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-dimethylaminopropyl) 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 hr to activate the carboxyl group. Afterwards, 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 hr. A solution containing 10 molar excess of PEG-MRF-DBD, PEG-TAP, and PEG-CPP with a mole ratio of 2:2:1 respectively was added drop-wise to the 10 nm AuNP solution and allowed to stir for 2 hr. The functionalized AuNPs (termed NanoScript) were filtered three times using a 10,000 MWC 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 Flour 594 (Invitrogen) fluorescent dye to TAP. Specifically, the free carboxy group on PEG-TAP was further conjugated to the Alexa Flour 594 Hydrazide dye via EDC/NHS coupling as described above.
**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 3.3a. 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 µl/min. To measure the rates of association (ka), dissociation (kd) 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.

**ADMSC Culture and Myogenic Differentiation:** The human adipose-derived mesenchymal stem cells (ADMSCs) (American CryoStem) and the 0.5 % FBS growth media were provided by American CryoStem. All cell culture was performed using the manufacture’s protocol. The ADMSCs were grown on fibronectin (2 µg/cm2, Millipore) coated culture dishes, and maintained at 37°C in a humidified incubator with 5% CO₂. For consistency, all experiments were carried out on cells between passages 2 to 4. For
myogenic differentiation experiments, ADMSCs were seeded in a 24-well plate at a density of 20,000 cells per well. After 24 hr, 10 µg/mL of NanoScript-MRF was incubated with the cells for 4 hr. Thereafter, the cells were washed twice with PBS and myogenic media was added. The myogenic growth media was comprised of 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 hr and washed twice with PBS and then fresh myogenic media was added. The cells were allowed to differentiate for 7 days in myogenic growth media, which was replaced with fresh media 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)

**ICP-OES Measurement:** After ADMSCs were seeded in a 24-well plate (20,000 cells/well), 10 µg/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 µL of lysis buffer was added. Then 250 µL 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, 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 μm, 3.0× 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 AuNPs was calculated using its excitation coefficient.

**Immunocytochemistry:** To investigate the nuclear localization of the dye-labeled NanoScript in ADMSCs, the media was removed and the cells were fixed for 15 minutes in formalin (Sigma) followed by two washes with PBS. The nucleus was stained with DAPI (Life Technologies) for 30 minutes 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 minutes and then washed twice with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and non-specific binding was blocked with 5% normal goat serum (NGS, Life Technologies) in PBS for 1 hr 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 washing three times with PBS, the samples were incubated for 1 hr at room temperature in a solution of anti-mouse secondary antibody labeled with Alexa Flour 647 (1:100, Life Technologies), anti-rabbit secondary antibody labeled with Alexa Flour 546 (1:100, Life Technologies), and DAPI (1:100, Life Technologies), in PBS containing 10% NGS. After washing three times, all the samples were imaged using a Nikon T2500 inverted fluorescence microscope.

**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 are below.

<table>
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<th>Target</th>
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<th>Reverse Primer (5’ – 3’)</th>
<th>Expected Size (bp)</th>
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3.3 RESULTS AND DISCUSSION

3.3.1 Design and Characterization of NanoScript-MRF

The NanoScript platform, designed to replicate the multi-domain structure of natural TFs, is comprised of 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 (AuNP) that acts as the linker domain to tether these components.

The first small molecule that we synthesized was a hairpin polyamide DBD, which is comprised of 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.\(^{12, 13}\) 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 (Figure 3.2).\(^{14}\) Hence, using an established solid phase synthesis procedure,\(^{14}\) 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 3.3a). 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 3.3b). Then, to
represent the AD, we designed a peptide referred to as a trans-activation peptide (TAP), which has been shown to act as a potent activator and inducer of transcriptional activity. Finally, an established cell penetrating peptide (CPP) was designed to facilitate nuclear localization of NanoScript.

**Figure 3.2: 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.'
In order for the NanoScript platform to be have 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. Furthermore, thiol-terminated PEG molecules enable functionalization of a
mixed monolayer on AuNPs.\textsuperscript{16, 18} Hence, we conjugated all three biomolecules (MRF-DBD, 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 3.4a, 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 3.4c). Using HPLC, the amount of ligands per gold nanoparticle was found to be 1297 (± 102) (Figure 3.5), which is in correlation to previous reports.\textsuperscript{19} Furthermore, the solubility of NanoScript-MRF in physiological environments was evaluated by incubation in cell culture media for seven days. Specifically, we found that the NanoScript-MRF maintained its monodisperse properties as compared to Day 0 (Figure 3.6).

![Figure 3.4: 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) The 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.](image-url)
Figure 3.5: 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 held 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.¹ The ligands are thiol-PEG-peptide molecules which were functionalized on the nanoparticle via the thiol group.

Figure 3.6: 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.

<table>
<thead>
<tr>
<th>Ligands/AuNP (10nm)</th>
</tr>
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<tbody>
<tr>
<td>1297 ± 102</td>
</tr>
</tbody>
</table>
3.3.2 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 nuclear membrane.\textsuperscript{16, 20} The second feature is that the hydrodynamic diameter is smaller than 44 nm, which is the size of the nuclear pore.\textsuperscript{20} 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 adipose-derived mesenchymal stem cells (ADMSCs). In order to calculate the concentration of NanoScript to be incubated with the ADMSCs, we performed a dose-dependent cell viability assay which revealed that the highest concentration with the highest viability is 10 μg/mL, and hence, we used this concentration for all subsequent experiments (Figure 3.7). 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 Flour 568 dye (attached onto NanoScript) and DAPI (nucleus staining) (Figure 3.8).
In order to establish the NanoScript platform for stem cell differentiation, we evaluated NanoScript’s ability to genetically reprogram ADMSCs into muscle cells. 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. Hence, we predicted that by activating MRFs in ADMSCs, particularly *MyoD* because it has been established as the most prominent myogenesis gene, using NanoScript-MRF,
we can guide their differentiation to generate muscle cells. Adopting a differentiation timeline from a previous report, ADMSCs were treated with NanoScript-MRF by incubating them in the culture media, and then a myogenic media was introduced to support muscle growth (Figure 3.9a). After seven days, expression of muscle-specific markers and phenotype changes were analyzed. As evidenced by the expression of *myogenin*, a ubiquitous myogenic marker, and *myosin*, a distinct muscle marker, we observed generation of myocytes, which are elongated, tubular muscle fiber cells, using the NanoScript-MRF platform (Figure 3.9b). This result was compared to the untreated control, which also included replacement with myogenic media, and showed minimal

*Figure 3.8: NanoScript Enters ADMSCs and Localizes in the Nucleus.* (a-d) 48 hr post-transfection, fluorescence microscopy was used to 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 is uptaken and is localized inside the nucleus. (Scale bar = 20 μm)
Figure 3.9: 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 multi-nucleated 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)
expression of myogenic markers (Figure 3.9b). Additional control experiments evaluated the contribution of each domain on NanoScript. Because these controls 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 (Figure 3.10, Figure 3.11). 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 3.9c).

This myogenic differentiation was further confirmed using qPCR to quantify the activation of muscle-specific genes. Two distinct intermediate myogenic genes, desmin and enolase, along with all four MRFs were effectively activated in cells treated with NanoScript-MRF, as compared to the control (Figure 3.12a). Furthermore, the late-stage myocyte marker, myosin heavy chain (MYH1), was up-regulated by about 28-fold (Figure 3.12a). 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 ADMSC’s were suppressed and significantly down-regulated, thus indicating that differentiated cells no longer genetically-resemble ADMSCs
Moreover, cell viability assays indicated the viability of generated muscle cells to be 97.6±1.1% (statistics from three independent experiments), as compared to the untreated control. Furthermore, we tested in NanoScript had any adverse effects on the DNA, such as inducing double strand breaks,\cite{16} using a commercially-available staining kit.
Figure 3.11: 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 3.12: NanoScript-MRF Initiates Transcription and Activation of Myogenic Genes. (a) After seven 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 stemness 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.
We found that NanoScript does not generate double stand breaks (Figure 3.13).

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.

![Image of cell culture](image)

**Figure 3.13: 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 μm).

### 3.3.4 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 seven days, we observed a dose-dependent increase of MyoD gene expression (Figure 3.14a); 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.

**Figure 3.14: NanoScript Outperforms TFs and is Excreted After Inducing Myogenesis.** (a) The 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 3.5a. (b) Schematic representation of NanoScript being uptaken 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). Based on microscope visualization, the cells stopped proliferating during the differentiation period, which is a phenomenon supported by literature. 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 to the highest peak on Day 2). Error bars are from three independent experiments.
3.3.5 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 3.14b). 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 3.14c). This result as in agreement with previously reports that demonstrate exocytosis of gold nanoparticles.16,23

3.4 CONCLUSION

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, our NanoScript-MRF mimicking the functions of natural TFs could be an alternative method for stem cell-based regenerative medicine. As 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 non-viral manner.

As the NanoScript platform regulates genes in a non-integrative approach, it is a highly attractive tool for conventional gene-regulating methods. As such, we envision that the multi-functional 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, or epigenetic modification molecules 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 (MRI) and multi-
faceted 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.

3.5 REFERENCES FOR CHAPTER 3


Chapter 4

Integrating Epigenetic Modulators onto NanoScript for Stem Cell Chondrogenesis

N-(4-Chloro-3-(trifluoromethyl)phenyl)-2-ethoxybenzamide (CTB) is a small molecule that functions by altering the chromatin architecture to modulate gene expression. We report a new CTB derivative with increased solubility and demonstrate CTB's functionality by conjugating it on the recently established NanoScript platform to enhance gene expression and induce stem cell differentiation. Modifying NanoScript with CTB will more closely replicate the TF structure and enhance CTB functionality and gene expression. To this end, we first conjugated CTB onto NanoScript and initiated a time-dependent increase in histone acetyltransferase activity. Next, because CTB is known to trigger the pathway involved in regulating Sox9, a master regulator of chondrogenic differentiation, we modified a Sox9-specific NanoScript with CTB to enhance chondrogenic gene activity and differentiation.
4.1 INTRODUCTION

Stem cell-based therapies and cellular reprogramming holds immense potential for regenerative medicine. Therefore, significant effort has been invested to develop methods for effectively controlling the differentiation of stem cells into specific lineages. One promising approach for enhancing gene expression and inducing stem cell differentiation involves epigenetic modification.1, 2 Epigenetic modification refers to changes in gene activity and expression by modifying chromatin structure rather than altering the DNA sequence.3 The eukaryotic genome is packed into chromatin, in which the DNA wraps around proteins called histones, and gene expression is regulated in part by the dynamics of chromatin structure.4 Moreover, epigenetic modification plays a critical role in enabling transcription factor (TFs) proteins, which are master regulators of gene expression, to regulate chromatin structure for inducing transcriptional activity.5

Specifically, epigenetic modification involves DNA histone deacetylation and acetylation, which serves to regulate the chromatin structure. One class of epigenetic modification molecules is called histone acetyltransferases (HATs), which functions to by weakening the association of DNA with histones to make the DNA more accessible for transcription and gene activation (Figure 4.1a). Moreover, HATs play an important role for regulating the transcriptional network of stem cells.6 Of the many HAT activator small molecules, a molecule called CTB [N-(4-Chloro-3-trifluoromethyl-phenyl)-2-ethoxy-
benzamide has been demonstrated to be effective in regulating HAT activity. CTB functions by triggering the p300 signaling pathway, which in turn, influences chromatin structure by facilitating the assembly of chromatin remodeling proteins to DNA binding sites (Figure 4.1a). As a result, CTB enhances p300 HAT-dependent transcriptional activation and regulates expression of p300-related genes. One such gene regulated by the p300 signaling pathway is Sox9, which plays a critical role for initiating and inducing chondrogenic differentiation of stem cells.

**Figure 4.1: Schematic Representation of CTB’s Function in Initiating Chondrogenesis.**

(a) If DNA (black line) is tightly coiled around histone proteins (blue cylinders), transcriptional activity and subsequent gene expression is inhibited. But activation of histone acetyltransferase (HAT) via the CTB molecule loosens the DNA to make target binding sites available for transcription. (b) The CTB is functionalized on the NanoScript platform, and the resulting construct is delivered to adipose-derived mesenchymal stem cells (AMDSCs) to enhance overexpression of targeted genes. The NanoScript construct is also modified with additional small molecules that target and overexpress the Sox9 gene. (c) Activation of the Sox9 gene in AMDSCs guides enhanced differentiation into the chondrogenic lineage.
We have recently demonstrated a new gene-regulating platform called NanoScript, which mimics the fundamental structure and function of transcription factor proteins. NanoScript was demonstrated to effectively induce gene expression of both a reporter plasmid and endogenous genes on native DNA in a non-viral manner, thus establishing NanoScript as a particularly attractive platform for applications requiring gene regulation. NanoScript was designed to mimic the two fundamental domains on TFs: the activation domain (AD) and the DNA-Binding Domain (DBD). The DBD seeks and binds to target gene sequences on DNA while the AD recruits a unit called the transcriptional basal machinery to initiate transcriptional activity. These two domains function synergistically to induce targeted gene expression. Through a simple modification of the DBD, NanoScript can be designed to target and activate the Sox9 gene. Moreover, it is interesting to note that studies have shown CTB to trigger the p300 pathway, and the p300 pathway has also been demonstrated to recruit the transcriptional basal machinery to initiate transcriptional activity. Therefore, we hypothesize that by modifying NanoScript with a CTB molecule, an AD molecule, and a Sox9 targeting molecule, we can utilize the NanoScript platform to achieve enhanced Sox9 expression in stem cells to accelerate their chondrocyte differentiation pathways.

Herein, we developed the NanoScript platform combined with an epigenetic modulator (e.g. CTB) and a Sox9 targeting molecule to activate Sox9 expression in adipose-derived mesenchymal stem cells (ADMSCs) to induce enhanced chondrogenic differentiation
(Figure 4.1b). First, we introduce a new functional CTB derivative modified with a thiol-PEG linker molecule to increase solubility and enable conjugation on NanoScript. Second, when the NanoScript platform, originally designed to mimic transcription factor proteins, is modified with CTB, we developed NanoScript to more closely mimic TF protein structure because TFs contain an epigenetic modification domain. Finally, when a CTB-modified NanoScript specific for Sox9 (termed NanoScript-Sox9) is delivered to ADMSCs, enhanced gene expression of Sox9 and subsequent chondrogenic differentiation was observed.

The NanoScript platform was designed and constructed to replicate the fundamental structure of multi-domain transcription factor proteins. Specifically, in order to replicate the structure of TF proteins, we designed NanoScript with six components: 1) the CTB molecule that mimics the epigenetic modification domain and functions to initiate HAT activity for enhanced gene expression,7 2) a hairpin polyamide molecule that mimics the DNA-Binding Domain (DBD) and functions to target and bind to the Sox9 gene,15,18 3) a transactivation peptide that mimics the activation domain (AD) and functions to initiate transcriptional activity,19 4) a membrane penetrating peptide to facilitate entry into the nucleus,20 5) polyethylene glycol (PEG)-based molecules to increase stability and solubility,21 and 6) a gold nanoparticle (AuNP) to tether these molecules onto a single construct.22 Assembling these individual small molecules on an AuNP enables the resulting NanoScript platform to emulate TF protein structure and function.
4.2 METHODS

**Peptide Sequences for STF Molecules:** The transactivation peptide, having a sequence of OH-CGSDALDDFDLDMLGSDALDDFDLDMLGS-NH₂, was purchased from Invitrogen. The cell penetrating peptide (CPP), having a sequence of NH₂-CALNNAGRKRRQRRR-OH, was purchased from GenScript.

**Synthesis of Hairpin Polyamides:** All machine-assisted polyamide syntheses were performed on a PSSM-8 peptide synthesizer (Shimadzu, Kyoto) with a computer-assisted operation system at 40 mg of Fmoc-β-wang resin (ca. 0.6 mmol/g, 100~200 mesh) by Fmoc solid-phase chemistry. Reaction cycles were as follows: deblocking steps for 4 min x 2, 20% piperidine in DMF; coupling step for 60 min, corresponding carboxylic acids, HCTU (88 mg), diisopropylethylamine (DIEA) (36 μL), 1-methyl-2-pyrrolidone (NMP); washing steps for 1 min x 5, DMF. Each coupling reagents in steps were prepared in NMP solution of Fmoc-Py-COOH (77 mg), Fmoc-Im-COOH (77 mg), Fmoc-PyIm-COOH (70 mg), Fmoc-β-COOH (66 mg), Fmoc-γ-COOH (69 mg). All other couplings were carried out with single-couple cycles stirred by N₂ gas bubbling. Typically, resin (40 mg) was swollen in 1 mL of NMP in a 2.5-mL plastic reaction vessel for 30 min. 2-mL plastic centrifuge tubes with loading Fmoc-monomers with HCTU in NMP 1 mL were placed in programmed position. All lines were washed with NMP after solution transfers. After the completion of
the synthesis by the peptide synthesizer, the resin was washed with DMF (1 mL x 2), methanol (1 mL x 2), and dried in a desiccator at room temperature in vacuo.

To synthesize the AcPyPyPy-β-PyImPy-γ-PyPyPy-β-PyImIm-β-NH₂ polyamide, a dried sample resin was cleaved with 0.5 ml of 3,3’-diamino-N-methyldipropylamine for 3 h at 45 °C. The reaction mixture was filtered, triturated from CH₂Cl₂-Et₂O, to yield Py-Im polyamide as a white-yellow crude powder. The crude was purified by HPLC (elution with trifluoroacetic acid and a 20-50% acetonitrile linear gradient (0-30 min) at a flow rate of 3.0 mL min⁻¹ under 254 nm) as white powder (3.5 mg, 8% for 15 steps). ESI-TOF-MS (positive) m/z calcd for C₉₁H₁₁₃N₃₄O₁₇₂⁺ [M+2H]²⁺ 977.45; found 977.44. The preparation of mismatched polyamide, AcPyPyPy-β-PyImPy-γ-PyPyPy-β-PyPyIm-β-NH₂, was used by similar procedure, and confirmed by HPLC and Mass. ESI-TOF-MS (positive) m/z calcd for C₉₂H₁₁₄N₃₃O₁₇₂⁺ [M+2H]²⁺ 976.95; f

**Synthesis of CTB:** All reactions were conducted in oven-baked glassware with magnetic stirring under nitrogen or argon atmosphere. All reagents and solvents (anhydrous) were purchased from Sigma-Aldrich, Acros Organics, Alfa Aesar and used without further purification. Analytical thin layer chromatography (TLC) was performed on silica gel matrix on TLC Al foil plates with fluorescent indicator 254 nm. Visualization was accomplished with UV light and iodine stain. Purification of reaction products was carried out by flash column chromatography over Teledyne Isco RediSep Rf silica gel (60
A, 40-60UM). The proton nuclear magnetic resonance (\(^1\)H-NMR) spectra were recorded on either a Varian-300 instrument (300 MHz), Varian-400 instrument (400 MHz), or Varian-500 instrument (500 MHz) spectrometer with a set temperature of 25°C. Chemical shifts (δ) of the compounds were recorded in ppm corresponding to the internal standard, tetramethylsilane (TMS). NMR solvent standards were used as follows: for \(^1\)H-NMR, CDCl\(_3\) = 7.26 ppm. Data were reported as chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (J) in Hz, and integration. Mass spectroscopy was recorded on a Finnigan LCQ-DUO mass spectrometer.

**Synthesis of thiol-PEGylated CTB.** (i) Preparation of heterobifunctional PEG linker: (a) (a-1) NaH, allyl bromide, DMF, 25 h, (a-2) TsCl, DMAP, Et\(_3\)N, CH\(_2\)Cl\(_2\), 16 h. (ii) Synthesis of thiol-PEGylated CTB: (b) TFA/TFAA, acetone, 24 h; (c) 1, K\(_2\)CO\(_3\), acetonitrile,
80°C, 24 h; (d) 4-chloro-2-(trifluoromethyl)aniline, DMPU, n-BuLi, THF, 80°C, 2 h; (e) AIBN, thioacetic acid, toluene, 100-110°C, 16 h; (f) Et₃SO₄, K₂CO₃, acetone, 48 h; (g) TBACN, MeOH, CHCl₃.

**Compound 1: p-toluenesulfonyl tetraethylene glycol allyl ether.** Compound 1 was prepared according to literature with slight modification.²³, ²⁴ Tetraethylene glycol (32.11 g, 0.17 mol) was dissolved in 70 mL DMF and cooled to 0°C under inert atmosphere. NaH (60% in mineral oil, 1.32 g, 33 mmol) was added in portions. After addition, the reaction was stirred at room temperature for 1 h. Then, allyl bromide (4 g, 33 mmol) was added dropwise. The mixture was allowed to proceed overnight. Then, the reaction was quenched with water, followed by washing with hexane and extracting with diethyl ether (more than 10 times) to yield colorless liquid oil (intermediate compound). The intermediate compound was used for next step without further purification. To an ice-cold solution of the intermediate compound (12.61 g, 54 mmol) in 100 mL dichloromethane, p-toluenesulfonate (30.78, 0.16 mol) was added. Then, Et₃N (73.41 mL, 0.54 mol) was added dropwise at 0°C using addition funnel. The reaction was stirred overnight at ambient temperature. Upon completion, the reaction mixture was filtered and the precipitant was washed with aqueous saturated solution of NaHCO₃ and dried over MgSO₄. MgSO₄ was filtered out and the solution was concentrated *in vacuo* to yield brown crude mixture. The crude mixture was purified by column chromatography on silica gel using dichloromethane/ethyl acetate (4:1) as eluent to afford yellow liquid oil. Yield. 30%. ¹H-
NMR (300 MHz, CDCl₃): δ (ppm) 7.80 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.6 Hz, 2H), 5.98-5.83 (m, 1H), 5.31 – 5.14 (m, 2H), 4.16 (t, 2H), 4.01 (dt, J = 5.7, 1.4 Hz, 2H), 3.72 – 3.54 (m, 14H), 2.44 (s, 3H); MS (m/z): calculated for C₁₈H₂₈O₇S, 388.16; found, 411.12 for [M+Na]^+.

**Compound 2:** 5-hydroxy-2,2-dimethyl-4H-benzo[d-1,3]dioxin-4-one. Synthetic procedure of compound 2 was adopted from literature. To a suspension of 2,6-dihydroxybenzoic acid (3 g) in trifluoroacetic acid (28.8 mL), trifluoroacetic anhydride (18 mL) and acetone (3.6 mL) were added at 0°C. The suspension was warmed slowly to room temperature. After 24h, the clear yellow solution was observed, and then the volatiles were evaporated under reduced pressure. Saturated aqueous solution of NaHCO₃ was added to the residue. Then, the aqueous solution was extracted with three portions of ethyl acetate. The combined organic layers were washed with water and brine, and then dried over MgSO₄. The solvent was evaporated after filtrating MgSO₄ to afford a yellow crude mixture. Chromatography over silica gel with hexane/ethyl acetate (4:1) as the eluent gave white solid product. Yield. 35%. ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 10.34 (br, s, 1H), 7.41 (t, J = 8.3 Hz, 1H), 6.63 (dd, J = 8.5. 0.9 Hz, 1H), 6.44 (dd, J = 8.2, 0.9 Hz, 1H), 1.75 (s, 6H); MS (m/z): calculated for C₁₀H₁₀O₄, 194.06; found, 217.00 for [M+Na]^+.

**Compound 3:** 5-((3,6,9,12-tetraoxapentadec-14-en-1-yl)oxy)-2,2-dimethyl-4H-benzo[d-1,3]dioxin-4-one. Compound 3 was synthesized according to literature. 5-hydroxy-2,2-dimethyl-4H-benzo[d-1,3]dioxin-4-one 2 (0.45 g, 2.34 mmol) and p-
toluenesulfonyl tetraethylene glycol allyl ether 3 (1 g, 2.57 mmol) were reacted in 15 mL acetonitrile in the presence of K₂CO₃ (0.36 g, 2.57 mmol). The reaction mixture was refluxed under inert atmosphere. After 24h, the mixture was allowed to cool down to room temperature. The mixture was diluted with diethyl ether. The solid byproduct was filtered out. The precipitant was concentrated in vacuo. The crude mixture was chromatographed on silica gel column using dichloromethane/ethyl acetate (4:1) as eluent to yield yellow liquid product. Yield. 99%. ¹H-NMR (500 MHz, CDCl₃): δ (ppm) 7.40 (t, \( J = 8.3 \) Hz, 1H); 6.62 (dd, \( J = 8.5, 0.9 \) Hz, 1H); 6.54 (dd, \( J = 8.1, 0.9 \) Hz, 1H); 5.96 – 5.84 (m, 1H); 5.31 – 5.13 (m, 2H); 4.22 (t, \( J = 5.0 \) Hz, 2H); 4.00 (dt, \( J = 5.7, 1.4 \) Hz, 2H); 3.95 (t, \( J = 5.0 \) Hz, 2H); 3.84 – 3.56 (m, 12H); 1.68 (s, 6H); MS (m/z): calculated for C₂₁H₃₀O₈, 410.19; found, 433.05 for [M+Na]⁺.

**Compound 4:** 2-((3,6,9,12-tetraoxapentadec-14-en-1-yl)oxy)-N-(4-chloro-3-(trifluoromethyl)phenyl)-6-hydroxybenzamide. The synthesis followed the reported protocol.²⁹, ³⁰ To a solution of 4-chloro-3-(trifluoromethyl)aniline (1.92 g, 9.79 mmol) and DMPU (2.46 g, 19.19 mmol) in 25 mL THF was added n-BuLi (3.92 mL of 2.5 M in hexane, 9.79 mmol) at 0°C. The reaction mixture was stirred at room temperature for 30 mins. In a separate flask, compound 3 (0.8 g, 1.95 mmol) was dissolved in 25 mL THF. The solution of compound 3 was then added to the aniline solution. After addition, the reaction was proceeded at 80°C for 2 h. Water was added to quench the reaction. Then, the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with 10%
aqueous solution of hydrochloric acid, followed by water and brine, then dry over MgSO₄. After solvent evaporated, the crude mixture was purified by column chromatography (silica gel, 4:1 dichloromethane/ethyl acetate mixture) to yield yellow liquid product. Yield. 31%; ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 13.32 (s, 1H), 10.69 (s, 1H), 8.07 (s, 1H), 7.95 (d, J = 8.7, 1H), 7.48 (d, J = 8.7 Hz, 1H), 7.31 (t, J = 8.4 Hz, 1H), 6.67 (d, J = 8.4 Hz, 1H), 6.42 (d, J = 7.6 Hz, 1H), 5.99 - 5.81 (m, 1H), 5.30 – 5.13 (m, 2H), 4.34 – 4.28 (m, 2H), 4.06 – 3.96 (m, 4H), 3.77 – 3.71 (m, 2H), 3.63 – 3.50 (m, 10H). MS (m/z): calculated for C₁₅H₁₅ClF₃NO₇, 547.16; found, 570.1 for [M+Na]⁺.

**Compound 5:** S-(1-(2-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)-3-hydroxy phenoxy)-3,6,9, 12-tetraoxapentadecan-15-yl) ethanethioate. To a solution of compound 4 (0.34 g, 0.61 mmol) in 30 mL toluene was added AIBN (0.03, 0.18 mmol) and purged with argon. Thioacetic acid (0.23 mL, 0.18 mmol) was added. The solution was stirred at 100-110 °C overnight under argon atmosphere. Upon completion, toluene was evaporated. The residue was re-dissolved in fresh toluene, and the procedure was repeated for 3 times. The crude mixture was then loaded into chromatographic column over silica gel using dichloromethane, following by 15:1 and 10:1 dichloromethane/ethyl acetate mixture) to yield yellow liquid oil. Yield. 65%. ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 10.69 (s, 1H), 8.05 (d, J = 2.2 Hz, 1H), 7.97 (dd, J = 8.7, 2.3 Hz, 1H), 7.49 (d, J = 8.9 Hz, 1H), 7.32 (t, J = 8.3 Hz, 1H), 6.69 (dd, J = 8.4, 1.0 Hz, 1H), 6.43 (dd, 1H), 4.32 (t, J = 4.4 Hz, 2H), 4.02
(t, J = 4.7 Hz, 2H), 3.80 – 3.38 (m, 14H), 2.92 (t, J = 7.1 Hz, 2H), 2.31 (s, 3H), 1.91 – 1.74
(m, 2H); MS (m/z): calculated for C_{27}H_{33}ClF_{3}NO_{8}S, 623.16; found, 646.0 for [M+Na]^+.

**Compound 6:** \textit{S-}(1-\textit{2}-(\textit{4}-\textit{chloro-3-(trifluoromethyl)phenyl})\textit{carbamoyl})-3-\textit{ethoxy}
\textit{phenoxy})-3,6,9,12-tetraoxapentadecan-15-yl) ethanethioate. Following the protocol for
alkylation from literature\textsuperscript{29, 30}, a suspension of \textit{S-}(1-\textit{2}-(\textit{4}-\textit{chloro-3-}
(trifluoromethyl)phenyl)\textit{carbamoyl})-3-\textit{hydroxyphenoxy})-3,6,9,12-tetraoxapentadecan-
15-yl) ethanethioate (0.25 g, 0.4 mmol) and K\textsubscript{2}CO\textsubscript{3} (0.14 g, 0.99 mmol) in 10 mL acetone
was added Et\textsubscript{2}SO\textsubscript{4} (77.8 uL, 0.6 mmol). The reaction was stirred for 48h at ambient
temperature. The reaction was added aqueous saturated solution of NH\textsubscript{4}Cl and extracted
with diethyl ether. The combined organic layers were washed with brine and dried over
MgSO\textsubscript{4}. After filtration, solvent was evaporated under reduced pressure. The flash column
chromatography was used to purify the mixture using dichloromethane/ethyl acetate (4:1)
as eluent to afford yellow liquid oil. Yield. 84%. \textit{^1H-NMR} (500 MHz, CDCl\textsubscript{3}): \(\delta\) (ppm) 8.61
(s, 1H); 8.02 – 7.89 (m, 2H); 7.37 (d, \(J = 8.6\) Hz, 1H); 7.19 (d, \(J = 8.7\) Hz, 1H); 6.50 (m, 2H);
4.14 (t, 2H); 4.00 (quartet, \(J = 7.0\) Hz, 2H); 3.71 (m, 2H), 3.58 – 3.37 (m, 12H); 3.34 (t, \(J =
6.2\) Hz, 2H); 2.82 (t, \(J = 7.2\) Hz, 2H); 2.23 (s, 3H); 1.72 (quintet, \(J = 6.7\) Hz, 2H); 1.30 (t, \(J =
6.9\) Hz, 3H); MS (m/z): calculated for C\textsubscript{29}H\textsubscript{37}ClF\textsubscript{3}NO\textsubscript{8}S, 651.19; found, 674.7 for [M+Na]^+.

**Compound 7:** \textit{N-}(\textit{4}-\textit{chloro-3-(trifluoromethyl)phenyl})-2-\textit{ethoxy}-6-\textit{((15-mercapto-
3,6,9,12-tetraoxa-pentadecyl)oxy)benzamide}. The protocol for deprotection of
thioacetate was adopted from a reported procedure\textsuperscript{31}. Briefly, the thioacetate-protected PEG-CTB (21.3 mg, 0.03 mmol) was dissolved in 1 mL chloroform/methanol (1:1) mixture. The reaction vessel was purged with argon before addition of tetrabutylammonium cyanide (8.7 mg, 0.03 mmol). The reaction mixture proceeded for 24h. The reaction was monitored by mass spectrometry because the TLC spots of the starting materials and the product gave spots on the same Rf value. After reaction completed, the reaction mixture was partitioned with chloroform and water. The aqueous layer was then extracted with chloroform. The combined organic layers were washed with saturated NH\textsubscript{4}Cl solution and dried over MgSO\textsubscript{4}. The purification was employed through flash column chromatography on silica gel using hexane/ethyl acetate (1:1) as the eluting solvent. Yield. 83\%. $^1$H-NMR (400 MHz, CDCl\textsubscript{3}): $\delta$ (ppm) 8.56 (s, 1H), 7.94 (m, 2H), 7.38 (d, $J = 8.5$ Hz, 1H), 7.20 (t, $J = 8.3$ Hz, 1H), 6.50 (dd, $J = 8.3$, 3.9 Hz, 2H), 4.26 – 4.08 (m, 2H), 4.00 (quartet, $J = 6.9$ Hz, 2H), 3.78 – 3.65 (m, 2H), 3.62 – 3.28 (m, 14H), 2.49 (quartet, $J = 7.3$ Hz, 2H), 1.74 (quintet, $J = 6.4$ Hz, 2H), 1.35 – 1.23 (m, 4H); MS (m/z): calculated for C\textsubscript{27}H\textsubscript{35}ClF\textsubscript{3}NO\textsubscript{7}S, 609.18; found, 632.4 for [M+Na]$^+$.

**SPR Analysis:** The Surface Plasmon Resonance (SPR) assays were performed using a BIACORE X instrument as described before.\textsuperscript{9} Biotinylated hairpin DNAs were purchased from JBioS (Tokyo, Japan) and the sequences are shown in Figure 4.3a. Hairpin biotinylated DNA are immobilized to streptavidin-coated sensor chip SA as described before and SPR assays were carried out using HBS-EP buffer (10 mM HEPES pH 7.4,150
mM NaCl, 3mM EDTA, and 0.005 % Surfactant P20) with 0.1 % DMSO at 25 °C. A series of sample solutions with various concentrations (320 nM- 20 nM) were prepared in the buffer with 0.1 % DMSO and injected at a flow rate of 20 μl/min. The rates of association (ka), dissociation (kd) and the dissociation constant (KD), were analyzed with an appropriate fitting model using the BIAevaluation 4.1 program as mentioned before and the 1:1 binding with mass transfer was used for fitting the sensorgrams.

**Synthesis of Gold Nanoparticles:** The gold nanoparticles with an approximate diameter of 9 nm were prepared by the Ferns method of citrate reduction of HAuCl4 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 HAuCl4 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 color change, the solution was refluxed for another 5 minutes, then cooled to room temperature and filtered using a 0.45 μm syringe filter. Characterization of the AuNPs was performed using transmission electron microscopy (TEM) and dynamic light scattering (DLS), and 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.
Construction of NanoScript: NanoScript was constructed using a two-step method. First, the NH2 terminated STF molecules (hairpin polyamide [DBD], transactivation peptide [AD], and TAT peptide [CPP]) were conjugated to linker molecule, SH-PEG-COOH (Thiol-PEG-Carboxy 1KDa [Creative PEGWorks, PBL-8073]). The PEG molecule was dissolved into a 50 mM solution in ethanol. Then, 50 mM of 1-ethyl-3-(3-dimethylaminopropyl) 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 hr. Afterwards, a 5 mM solution of the STF molecule was added to the solution and allowed to react at room temperature for 2 hr. The solution was kept on a shaker during this time, and the percent yield for this EDC/NHS reaction was 96%. The three STF molecules conjugated with PEG (PEG-Sox9-DBD, PEG-AD, and PEG-CPP), were coated on 10 nm AuNPs. A solution containing 10 molar excess of 20% PEG-CPP, 30% PEG-DBD, 25% CTB, and 25% PEG-AD was added drop-wise to the AuNP solution and allowed to stir for 2hr. The functionalized AuNPs (termed NanoScript) were filtered three times using a 10,000 MCWO filter (Millipore) to remove unreacted molecule and to adjust the volume to the desired final concentration. The dye-labeled NanoScript, used for tracking intracellular localization of NanoScript, was constructed by conjugating Alexa Flour 594 (Invitrogen) fluorescent dye to the AD (the transactivation peptide). Specifically, the free carboxyl group on PEG-AD was further conjugated to the Alexa Flour 594 Hydrazide dye via EDC/NHS coupling as described above.
**ADMSC Culture and Chondrogenic Differentiation:** Commercially available human adipose-derived mesenchymal stem cells (ADMSCs) (ATCELLs™, American CryoStem) were expanded using the manufacture’s protocol using standard 0.5% FBS growth media. The ADMSCs were expanded on fibronectin (2 μg/cm², Millipore) coated culture dishes, and maintained at 37°C in a humidified incubator with 5% CO2. For consistency, all experiments were carried out on cells between passages 2 to 4. For chondrogenic differentiation experiments, ADMSCs were seeded in a 12-well plate at a density of 50,000 cells per well. After 24 hr, a 1 nM of NanoScript-Sox9 was incubated with the cells for 4hr, after which, the cells were washed twice with PBS and fresh commercially-available chondrogenic media (ACSelerate CY™ [media formulation is kept confidential by the company], American CryoStem) was added. After 48hrs, the ADMSCs were re-transfected with 1 nM of NanoScript-MRF for 4 hr, washed twice with PBS, and given fresh chondrogenic media. The cells were allowed to differentiate for 7 days with chondrogenic media changes every other day. All conditions and controls were transfected with 1 nM of the constructs.

**Immunocytochemistry:** To investigate the nuclear localization of the dye-labeled NanoScript in ADMSC cells, the media was removed and the cells were fixed for 15 minutes in formalin (Sigma) followed by two washes with PBS. The nucleus was stained with DAPI (Life Technologies) for 30 minutes and then washed with PBS three times. To investigate the extent of chondrogenic differentiation on Day 7, the media was removed and fixed
with formalin for 15 minutes and then washed twice with PBS. Cells were then
permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and non-specific binding was
blocked with 5% normal goat serum (NGS, Life Technologies) in PBS for 1 hour at room
temperature. To study the extent of chondrogenic differentiation, the primary mouse
antibody against Collagen II (1:200 dilution, Santa Cruz Biotechnologies) and the primary
rabbit antibody against Aggrecan (1:200 dilution, Abcam) 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 washing three times with PBS, the
samples were incubated for 1 hr at room temperature in a solution of anti-mouse secondary
antibody labeled with Alexa Flour 647 (1:100, Life Technologies), anti-rabbit secondary
antibody labeled with Alexa Flour 546 (1:100, Life Technologies), and DAPI (1:100, Life
Technologies), in PBS containing 10% NGS. After washing thrice, all the samples were
imaged using the Nikon T2500 inverted fluorescence microscope.

**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).
Primers sequences are in the table below.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer (5’ – 3’)</th>
<th>Reverse Primer (5’ – 3’)</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>CCCCTGCTATTTTCATCGACCC</td>
<td>GACACACGGCTCCACTTGAT</td>
<td>90</td>
</tr>
</tbody>
</table>
HAT Assay: To perform the HAT assay and quantify the increase of HAT activity, we purchased two specialized kits. The first is the Nuclear/Cytosol Fractionation Kit (BioVision K266-100) to collect and purify the nuclear extract. The second is the HAT Activity Fluorometric Assay Kit (BioVision K334-100) to quantify the HAT activity in the samples. Both kits were carried out using the manufacturer’s protocol.

4.3 RESULTS AND DISCUSSION

4.3.1 Development of an Epigenetic Modulator CTB

The CTB molecule interacts with the p300 protein, which in turn, interacts with lysine amino acids on histone proteins. This results in reduced electrostatic attraction between the histone and the DNA, which causes the DNA to decrease its affinity, thus enabling more exposed binding sites for transcription. We carefully synthesized a modified derivative of the CTB in order to increase its solubility in physiological environments while preserving its functionality. Particularly, CTB binds to the target through hydrogen bonding and hydrophobic interactions, while the position of –Cl and –CF3 have the strongest influence in binding affinity (Figure 4.2a). Moreover, it has been demonstrated that the ortho position on the aromatic hydrocarbon does not play a critical role in overall binding.
Therefore, we modified the ortho position with a PEG-thiol chain for increasing solubility in physiological environments (via five –CH₂CH₂O motifs) and for enabling conjugation onto the gold nanoparticles (via the –SH motif) (Figure 4.2a). In doing so, we synthesized a CTB analog (see SI for the synthetic procedure).

**Figure 4.2: Function of CTB is Enhanced when Conjugated on NanoScript.** (a) The CTB molecule is modified with a thiol-PEG chain and functionalized on NanoScript. The remaining components on NanoScript include the AD which recruits endogenous factors to initiate transcription, the DBD (refer to Figure S1 for structure details) which targets and binds to the target Sox9 sequence on DNA. The cell penetrating peptide enables NanoScript to permeate the plasma and nuclear membrane. (b) The HAT activity of this NanoScript-Sox9 construct was quantified and showed increased in HAT activity across three time points (Day 1, 2, 3). Controls that include only the AD (without CTB), only CTB (without AD), or unconjugated CTB in media, showed comparatively less HAT activity. All conditions and controls were transfected with 1 nM of the constructs. The increase of HAT for all samples was determined by normalizing to the untreated control. Standard error is from three independent trials.
4.3.2 Development of Chondrogenic-specific Biomolecules

Then we synthesized the hairpin polyamide specific for the Sox9 gene. The hairpin polyamide, which acts as the DBD, consists of imidazole and pyrrole motifs that bind to the G-C and A-T base pairs on the DNA respectively with nanomolar affinity.\(^3^4\) The Sox9 gene has a consensus promoter sequence of 5'-ACAATGG-3';\(^3^5\) thus we designed a hairpin polyamide to target this sequence (Figure 4.3a). Reported protocols using solid phase synthesis enabled us to synthesize the hairpin with a sequence of PyPyPy-β-PyImPy-γ-PyPyPy-β-PyImIm-β-Dp-NH\(_2\) that is complementary to the target Sox9 gene.\(^3^6\) In-vitro binding association studies were performed using Surface Plasmon Resonance (SPR) to verify the binding affinity of the Sox9 polyamide. Results showed a high sequence specific binding affinity of the Sox9 polyamide to the target sequence as compared to controls, which included scrambled polyamides and scrambled DNA sequences (Figure 4.3b).

The third molecule is the transactivation peptide, which emulates the activation domain (AD) and functions to recruit the transcriptional basal machinery required to initiate transcription.\(^3^7\) This peptide has been demonstrated to be a potent inducer of transcriptional activity and was specially designed in the D-form to resist intracellular degradation.\(^1^9\) The fourth molecule is the cell penetrating peptide to enable plasma membrane and nuclear membrane penetration. Specifically, we used a modified TAT peptide, which has been reported to shuttle gold nanoparticles into the nucleus.\(^2^0\)
4.3.3 Construction of NanoScript

These molecules (hairpin polyamide, transactivation peptide, and the TAT peptide) were conjugated to a PEG-thiol molecule (21 chain PEG) via EDC/NHS coupling, which enables functionalization onto gold nanoparticles. Gold nanoparticles were particularity
chosen because of their high biocompatibility, non-toxic effect in cells, and most importantly, because of their multifunctional surface that enables conjugation of multiple molecules on a single nanoparticle. After functionalizing 10 nm gold nanoparticles with these PEG-terminated molecules, along with the CTB, the resulting NanoScript platform [termed NanoScript-Sox9] targeted the Sox9 gene (Figure 4.2a). Characterization of NanoScript-Sox9 revealed a successive shift in the UV absorbance indicative of functionalization (Figure 4.4) and the hydrodynamic diameter was found to be 57.9 nm (Figure 4.5).

Figure 4.4: Small Molecules Conjugated on NanoScript-Sox9. Unmodified, citrate-stabilized 10 nm gold nanoparticles (AuNPs) show a characteristic peak at 517 nm. After functionalization with the small molecules, the successive shift in the absorbance peak to 532 nm suggests attachment of biomolecules on the AuNP surface.
After constructing the NanoScript-Sox9, we first wanted to ensure successful nuclear localization, which is an essential criterion because transcriptional activity occurs only in the nucleus. Hence, the NanoScript-Sox9 was labeled with a fluorescent dye (Alexa Flour 594) and transfected into adipose-derived mesenchymal stem cells (ADMSCs). After 24 hours, fluorescence imaging revealed that NanoScript-Sox9 was able to enter and localize within the nucleus of 51.6% of the total cells. *(Figure 4.6).*

### 4.3.5 NanoScript Induces HAT Activity

After ensuring nuclear uptake, we evaluated the function of the CTB molecule on the NanoScript-Sox9. It is well known that CTB initiates HAT activity. The effect of PEG modification on CTB was tested, and both the unmodified CTB and PEG-modified CTB showed similar HAT activity *(Figure 4.7)*; thus indicating that the modified CTB retained its functionality. Moreover, the function of CTB conjugated to the nanoparticle was tested, and revealed that increasing CTB amounts on the nanoparticle resulted in increasing HAT

<table>
<thead>
<tr>
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<th>Hydrodynamic Diameter (nm)</th>
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<tbody>
<tr>
<td>Unmodified AuNPs</td>
<td><strong>9.8 ± 0.3</strong></td>
</tr>
<tr>
<td>NanoScript-Sox9</td>
<td><strong>57.9 ± 8.1</strong></td>
</tr>
</tbody>
</table>

*Figure 4.5: Hydrodynamic Diameter of NanoScript-Sox9.* Using dynamic light scattering, the hydrodynamic diameter of NanoScript-Sox9 was determined to be 57.9 nm.
activity (Figure 4.8). Then we tested various concentrations of NanoScript-Sox9 and observed a concentration dependent increase of HAT activity (Figure 4.9). Next, NanoScript-Sox9 induced HAT activity was evaluated various time points. Remarkably, the
Figure 4.7: Modified CTB Retains HAT Functionality. To ensure that PEG modification of CTB does not influence its function of inducing HAT activity, we compared HAT activity of unmodified CTV versus PEG-modified CTB. After 48 hours, the HAT activity of both molecules was almost similar; thus indicating that modification to the ortho-position on CTB does not affect the functionality of the molecule.

Figure 4.8: Effect of Gold Nanoparticle CTB Ratios on HAT Activity. The ratio of CTB on the gold nanoparticle (AuNP) can play a role on HAT activity. When the ratio of CTB was varied, we observed an increase in HAT activity in correlation to increasing CTB ratios.
NanoScript-Sox9 with both the AD and CTB induced maximal HAT activity in all the time points compared to the controls, which included a free CTB molecule and NanoScript-Sox9 that lacked either the CTB or AD (Figure 4.2b). While this result shows induction of HAT activity when the CTB or AD are individually on NanoScript, the combined effect of CTB and AD on a single NanoScript showed maximum increase in HAT activity.

### 4.3.6 NanoScript Enhances Chondrogenic Differentiation

After determining that both CTB and AD would be required on a single nanoparticle to induce maximal HAT activity, we focused on overexpressing the Sox9 gene in ADMSCs to enhance chondrogenic differentiation. ADMSCs were chosen as a model cell line.
because they are excellent multipotent stem cells with a differentiation capacity into muscle, cartilage, bone, and neuronal cells.\textsuperscript{38-41} Moreover, Sox9 was chosen as a target gene because it known to not only control chondrogenic differentiation,\textsuperscript{13} but its expression is regulated by both the p300 pathway and conventional ADs.\textsuperscript{11} Therefore, we expected to observe an enhancement of Sox9 expression and other chondrogenic-related genes, when NanoScript-Sox9 is delivered to ADMSCs.

NanoScript-Sox9 and chondrogenic differentiation media was incubated with ADMSCs on Day 0 and Day 2. After seven days, the ADMSCs were evaluated for expression of distinct chondrogenic factors, Aggrecan and Collagen II, which were specifically chosen because they are prominent chondrogenic markers that are expressed based on the progression of chondrogenic differentiation. After the ADMSCs were fixed and stained fluorescence imaging revealed that the condition treated with NanoScript-Sox9 (having both CTB and AD) showed enhanced expression of Aggrecan and Collagen II as compared to controls (Figure 4.10). Furthermore, the fluorescence intensity of Aggrecan and Collagen II expression from these images was quantified and showed a remarkable trend that confirmed maximal expression when both CTB and AD are present on NanoScript-Sox9 (Figure 4.10), with high cell viability (Figure 4.11). Even though all conditions and controls were treated identically with differentiation media, maximum expression was observed when CTB and AD were both present on NanoScript-Sox9, thus suggesting enhanced chondrogenic differentiation.
To further quantify the genetic expression patterns of chondrogenic genes in NanoScript-Sox9 treated ADMSCs, we performed qPCR. Analysis showed that...
NanoScript-Sox9 enhanced expression of all three distinct genes, Sox9, Aggrecan, and Collagen II, as compared to controls and NanoScript lacking either the AD or CTB (Figure 4.10e). Another control experiment, of incubating NanoScript-Sox9 (w/o CTB) with free CTB, suggests that CTB has maximal function when attached to the gold nanoparticle (Figure 4.12). Even though the NanoScript-Sox9 was designed to target the Sox9 gene, which showed the highest expression among the three genes, Sox9 has been demonstrated to be sufficient to trigger downstream chondrogenic genes Aggrecan and Collagen II.

4.4 CONCLUSION

Taken together, the results from our three experiments (HAT assay, immunofluorescence, and qPCR) indicate that the presence of both CTB and AD on
NanoScript initiates maximal HAT activity and expression of chondrogenic genes. One possible hypothesis for this result is that the CTB and AD initiate HAT activity through different pathways, because previous reports demonstrate that CTB initiates HAT activity through the p300 pathway, while the AD (specifically the transactivation peptide) initiates HAT activity by recruiting several factors such as SAGA and NuA4. Hence, it is possible that because two gene activation pathways were induced, we observed an enhanced synergistic gene expression when both CTB and AD were present on NanoScript. However, the exact interplay between these signaling pathways is unknown and we will attempt to elucidate the mechanisms in future studies.

Figure 4.12: Influence of Free CTB on Gene Expression: The NanoScript-Sox9 (w/o CTB) with free CTB was incubated in ADMSCs and qPCR performed after 7 days to evaluate if free CTB can induce gene expression without being attached to the nanoparticle. The expression of all three chondrogenic genes, Sox9, Aggrecan, and Collagen II, are similar to that of NanoScript-Sox9 (w/o CTB) as shown in Figure 4.10c. This indicates that for enhanced gene expression, the CTB must be conjugated to the nanoparticle.
In summary, the recently developed NanoScript platform was modified with an epigenetic modification molecule, CTB, which regulates histone proteins and enhances gene expression through the HAT-dependent p300 pathway. Conjugation of CTB on NanoScript not only enabled NanoScript to move a step closer in mimicking natural transcription factors (which have epigenetic regulatory domains), but also enhanced the gene expression capabilities of NanoScript. First, the role and function of CTB on NanoScript was evaluated by measuring HAT activity, and revealed that CTB is effective in inducing HAT activity. Moreover, we observed that NanoScript (with both AD and CTB) showed the greatest HAT activity, which is possibly due to the fact that the AD and CTB triggered two distinct pathways for regulating HAT activity. Next, because NanoScript was designed to be specific for the Sox9 gene, which is the master regulator of chondrogenesis, we developed the NanoScript-Sox9 to induce chondrogenesis. When NanoScript-Sox9 was incubated with ADMSCs, we confirmed that NanoScript-Sox9 activates expression of chondrogenic markers through fluorescence imaging and qPCR.

In conclusion, our NanoScript-based transcriptional activator can have a significant impact in the field of stem cell biology and cellular reprogramming. Through this proof-of-concept demonstration, we have established that NanoScript is an effective and tunable platform that can manipulate targeted genes that are critical for stem cell differentiation in a non-viral manner. Moreover, by modifying NanoScript with epigenetic modification molecules such as HAT activators (e.g. CTB), we optimized NanoScript to be more effective.
in regulating gene expression. As a result, we are confident that NanoScript can be utilized for other applications requiring non-viral and effective gene regulation in stem cell biology and cellular reprogramming.

4.5 REFERENCES FOR CHAPTER 4


Even though gene repression is a powerful approach to exogenously regulate cellular behavior, developing a platform to effectively repress targeted genes, especially for stem cell applications, remains elusive. Herein, we introduce a nanomaterial-based platform that is capable of mimicking the function of transcription repressor proteins to downregulate gene expression at the transcriptional level for enhancing stem cell differentiation. We developed the NanoScript platform by integrating multiple gene repression molecules with a nanoparticle. First, we show a proof-of-concept demonstration using a GFP-specific NanoScript to knockdown GFP expression in neural stem cells (NSCs-GFP). Then, we show that a Sox9-specific NanoScript can repress Sox9 expression to initiate enhanced differentiation of NSCs into functional neurons.
5.1 INTRODUCTION

Stem cell differentiation and cellular reprogramming is fundamentally regulated through a process known as gene regulation.\textsuperscript{1} Gene regulation is an inherent cellular mechanism through which gene expression is either increased or decreased, and this has a direct impact on cellular behavior such as proliferation, migration, and differentiation.\textsuperscript{2} There are two types of gene regulation: 1) gene activation, which refers to an increase in the expression levels of a targeted gene, and 2) gene repression, which refers to a decrease in the expression levels of targeted genes.\textsuperscript{3}

Initiation of transcription of genetic information.\textsuperscript{4} Once TFs bind to their target gene, the gene can either be activated or repressed, depending on which domains are present on the TFs. A typical TF contains three fundamental domains: 1) a DNA-Binding Domain (DBD) which is sequence-specific and binds to target sequences, 2) a nuclear localization domain to enable the TF proteins entry inside the nucleus, and 3) either an activation domain or a repression domain (RD). If an activation domain is present on the TF, then the targeted gene will be transcribed and gene expression will be upregulated,\textsuperscript{5,6} and if a RD is present on the TF, then the target gene will be repressed and gene expression will be downregulated.\textsuperscript{7}

We recently developed a nanomaterial-based platform called NanoScript, which was designed to mimic the fundamental structure and function of TF activator proteins.\textsuperscript{8,9}
NanoScript was designed by attaching specific small molecules on a nanoparticle. These small molecules emulate the function of individual domains on TF proteins, and when multiple small molecules are assembled together on a single nanoparticle, the resulting NanoScript platform can mimic the function and structure of natural TF proteins. NanoScript is a platform with interchangeable components that can be modified depending on the desired application. Our previous NanoScript included a series of small molecules to mimic a subset of TF activator proteins. However, even though there are reports of small molecule-based approaches for transcriptional gene knockdown in \textit{in vitro} systems,\textsuperscript{10, 11} there have been no previous reports to either demonstrate NanoScript’s capability to mimic TF repressor proteins or to develop a nanomaterial-based platform that can effectively repress genes at the transcriptional level. We speculate that by modifying our NanoScript platform with repression-specific small molecules, we can design NanoScript to mimic transcriptional repressor proteins for effectively downregulating genes to induce stem cell differentiation.

Herein, we developed the NanoScript platform to effectively mimic the fundamental structure and gene-silencing function of TF repressor proteins. In order to emulate the function of each domain on natural TF repressor proteins, NanoScript was constructed by assembling multiple gene repression molecules, which function to inhibit and block the recruitment of factors to the DNA binding site to prevent gene expression, together on a multifunctional nanoparticle (\textbf{Figure 5.1a,b}). We performed a proof-of-concept
experiment by successfully repressing endogenous expression of green fluorescence protein (GFP) in neural stem cells. Moreover, NanoScript was utilized to repress the neuro-specific gene Sox9 in neural stem cells which induced their differentiation into neurons (Figure 5.1c). The primary advantage of our multifunctional NanoScript platform over conventional approaches is its ability to tether multiple repressor molecules, which function through different mechanisms, on a single nanoparticle to synergistically repress gene expression.

5.2 METHODS

**Synthesis of Peptides:** The corepressor peptide, with a sequence of NH$_2$-GWRPW-OH, was purchased from GenScript. The membrane penetrating peptide (MPP), with a sequence of NH$_2$-CALNNAGKRRQRRR-OH, was purchased from GenScript.

**Synthesis of GFP and Sox9 Hairpin Polyamides:** All hairpin polyamides were synthesized by adopting our previously published protocol. The Py-Im (pyrrole [Py], imidazole [Im]) polyamide synthesis was machine assisted 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 following synthetic procedure was performed: i) deblocking steps for 4 min
Figure 5.1: Schematic Representation of NanoScript-based Gene Repression. (a) When components of the transcriptional basal complex assemble on a target DNA sequence, such as the Sox9 promoter sequence, the corresponding gene is transcribed. (b) NanoScript-based gene expression is based on the synergistic effect of the DNA Binding Domain molecule for steric hindrance and the co-repressor molecule to disrupt the formation of the transcriptional basal complex on the target DNA sequence. (c) To demonstrate NanoScript-based repression in neural stem cells (NSCs), a GFP-specific NanoScript silences expression of GFP and a Sox9-specific NanoScript represses Sox9 to induce neuronal differentiation.
(2 times), 20% piperidine in DMF; ii) coupling step for 60 min, using 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 (5 times) in 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), and Fmoc-γ-COOH (69 mg), with stirring by N₂ gas bubbling. Typically, resin (40 mg) was swollen in 1 mL of NMP in a 2.5-mL plastic reaction vessel for 30 min. 2-mL plastic centrifuge tubes with loading Fmoc-monomers with HCTU in NMP 1 mL were placed in programmed position. After each solution transfer, all lines were washed with DMF. After the completion of the synthesis by the last acetyl capping on the peptide synthesizer, the resin was washed with DMF (1 mL, 2 times) and methanol (1 mL, 2 times), and dried in a desiccator at room temperature in vacuo.

To synthesize the GFP polyamide with sequence PyPyPy-β-PyPyIm-γ-PyPyPy-β-PyImPy-β-Dp (γ is γ-aminobutyric acid, β is β-alanine, and Dp is dimethylaminopropylamide), a dried resin was cleaved with 0.4 ml of 3,3'-diamino-N-methylidipropylamine for 3 h at 45 °C. Then the reaction mixture was filtered, triturated from CH₂Cl₂-Et₂O. This yielded a crude yellow powder. Purification of the crude 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 1.8 mL/min⁻¹
under 254 nm). ESI-TOF-MS (positive) m/z calculated for C_{92}H_{115}N_{33}O_{172}^{2+} [M+2H]^{2+} 976.76; found 976.94.

To synthesize the Sox9 polyamide with sequence, AcPyPyPy-β-PyImPy-γ-PyPyPy-β-PyImIm-β-NH₂, a dried sample resin was cleaved with 0.4 ml of 3,3’-diamino-N-methyldipropylamine for 3 h at 45 ºC. The reaction mixture was filtered, triturated from CH₂Cl₂-Et₂O, to yield Py-Im polyamide as a white-yellow crude powder. The crude was purified by HPLC (elution with trifluoroacetic acid and a 20-50% acetonitrile linear gradient (0-30 min) at a flow rate of 3.0 mL/min under 254 nm). ESI-TOF-MS (positive) m/z calculated for C_{91}H_{113}N_{34}O_{172}^{2+} [M+2H]^{2+} 977.45; found 977.44.

**Synthesis of Magnetic Core-Shell Nanoparticles:** Both the magnetic cores and core-shell particles were synthesized according to a previously reported protocol with slight modifications.¹²,¹³ The 10 nm zinc doped iron oxide magnetic cores were synthesized by thermal decomposition. In a 100 mL 3-neck round bottom flask, 1.5174 mmol Fe(Acac)₃, 0.4825 mmol FeCl₂, 0.3338 mmol ZnCl₂, 10 mmol 1,2-hexadecandiol, 6 mmol oleic acid, 6 mmol oleylamine and 20 mL tri-n-octylamine were mixed at 150°C under vacuum for 45 min. The vacuum was then removed and the temperature was increased to 200°C at a rate of 4°C per min under dry air for 2 hr, and then further increased to 300°C for 30 min at rate of 4°C per min also under dry air. The reaction mixture was cooled to room temperature and the particles were purified by dispersing the reaction mixture in ethanol.
and centrifuging at 10,000 rpm several times to produce a dry pallet. The particles were then dispersed and stored in chloroform. The particles were characterized by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer Nano ZS-90 and a Philips CM12 transition electron microscope (TEM).

The gold-coated magnetic nanoparticles (MNP@Au) were synthesized by reducing AuCl₃ on the 10 nm zinc-doped iron oxide magnetic cores. In a 50 mL 3-neck round bottom flask, 5 mgs of the 10 nm magnetic cores were mixed in 20 mL of tri-n-octylamine and heated to 60°C under vacuum for 10 min to evaporate the chloroform. Upon cooling the reaction mixture to room temperature, 0.3mmol (100 μL) of oleylamine and 60 μl of a 5 mg/300 μL stock solution of AuCl₃ were added and heated to 70°C under vacuum to evaporate the solvents, after which the temperature was increase to 150°C at a rate of 10°C per min under atmosphere for 4 hr. The reaction mixture was then cooled to room temperature and centrifuged at 10,000 rpm to collect the particles. The particles were purified with chloroform and magnetically decanted several times. The purified particles were dispersed and stored in minimal amount of chloroform.

The chloroform dispersed MNP@Au were rendered water soluble by carrying out a ligand exchange in TMAOH and citrate buffer. A TMAOH solution was prepared by dissolving 0.09 g of trisodium citrate in 15 mL of 1 M TMAOH. The previously prepared MNP@Au particles were added to the TMAOH solution and sonicated using a probe sonicator for 30 min. The solution was magnetically decanted and the particles were
purified several times using DI water and magnetic decantation and finally dispersed in DI water. The citrate-capped core-shell particles were verified using a Cary US UV-Vis spectrometer and a Philips CM12 TEM.

**Construction of NanoScript:** We developed the NanoScript platform using a two-step method. First, the three amine terminated biomolecules (WRPW peptide, MPP peptide, and GFP/Sox9 polyamides) were conjugated to a linker molecule, SH-PEG-COOH (Thiol-PEG-Carboxy 1KDa [Creative PEGWorks, PBL-8073]). 50 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC) (Sigma) and 50 mM of N-hydroxysuccinimide (NHS) (Acros Organics) was added to a solution of PEG (50 mM in EtOH), and placed on a shaker for 1 hr. A solution containing 10 molar excess of PEG-WRPW, PEG-MPP, and PEG-GFP/Sox9 polyamide (5 mM) with a mole ratio of 2:1:2 respectively was added drop-wise to the nanoparticle solution and allowed to stir for 2 hr. The functionalized nanoparticles (termed NanoScript) were filtered three times using a 10,000 MCFWO filter (Millipore) to remove unreacted molecules and to adjust the concentration.

The dye-labeled NanoScript, used for tracking intracellular localization of NanoScript, was constructed by conjugating the Alexa Flour 568 (Invitrogen) fluorescent dye to the PEG molecules. Specifically, the free carboxy group on PEG was conjugated to the Alexa Flour 568 Hydrazide dye via EDC/NHS coupling as described above.
Characterization of NanoScript was performed using multiple methods. The nanoparticle concentration and confirmation of functionalized was obtained using UV-visible absorption spectra (Varian Cary 5000 UV Vis-NIR Spectrophotometer). Using Dynamic Light Scattering (Malvern Zetasizer Nano-ZS90), we determined the hydrodynamic size of NanoScript. The shape and monodisperse properties of the nanoparticles was confirmed using transmission electron microscopy (TEM). The nanoparticles were drop-cast on the Holey-carbon grids (Electron Microscopy Sciences), allowed to dry overnight under vacuum, and subsequently imaged using a JEOL JEM-2010F high-resolution TEM operated at an accelerating voltage of 200 kV.

**SPR Binding Affinity:** The SPR assays were performed using a BIACORE X instrument. The biotinylated hairpin DNAs that is complementary for each of the polyamides were purchased from JBioS (Tokyo, Japan) (See table below for the biotinylated hairpin DNA sequences).

<table>
<thead>
<tr>
<th>For GFP Polyamide</th>
<th>3’-CCGAGGTATACAAACGGTTTTCCGTTGATACCTCCG-Biotin-5’</th>
</tr>
</thead>
<tbody>
<tr>
<td>For Sox9 Polyamide</td>
<td>3’-GCGTGTTACAAAGCTTTTGCTTGGTTACCACGC-Biotin-5’</td>
</tr>
</tbody>
</table>

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 performed 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 μl/min. To measure the rates of association ($k_a$), dissociation ($k_d$) and dissociation constant ($K_D$), data processing was performed with a fitting model using the BIAevaluation 4.1 program. The 1:1 binding with mass transfer was used for fitting the sensorgrams.

**Rat Neural Stem Cell (rNSC) Culture and GFP Knockdown:** The GFP-labeled rat neural stem cells (rNSCs) were purchased from Millipore and cultured according to the manufacturers’ protocol. The culture media specific for rNSCs was purchased from Millipore. All cells were maintained at 37°C in a humidified incubator with 5% CO₂. All experiments were carried on cells between passage 3 and 5.

For GFP knockdown studies, the cultured rNSCs (75,000/well, 12-well plate) were transfected with the NanoScript-GFP constructs (1 nM) in growth medium, and the cell culture plates were placed on the Nd-Fe-B magnetic plates (OZ Biosciences, France) for 15 mins. After 4 hr, the cells were washed twice with PBS and fresh rNSC media (now without bFGF to stop proliferation) was added. The rNSCs were transfected on Day 0 and Day 2, and the GFP knockdown levels was imaged and analyzed on Day 2 and Day 4. Fresh media was exchanged every other day.

**Human Neural Stem Cell (hNSC) Culture and Neuronal Differentiation:** The human neural stem cell (hNSC) line was purchased from Millipore and cultured according to the manufacturer’s protocol. All cells were maintained at 37°C in a humidified incubator with 5% CO₂. All experiments were carried on cells between passage 3 and 5. The hNSCs
were seeded in plates (125,000/well, 12-well plate; and 62,500/well, 24-well plate) in hNSC specific media (from Millipore) supplemented with basic fibroblast growth factor (bFGF, 20 ng/mL) and epidermal growth factor (EGF, 20 ng/mL), 24 hr prior to experimentation.

To induce neuronal differentiation, the NanoScript-Sox9 constructs (1 nM) were added to the hNSCs with the culture plates placed a magnetic plate as described above, and after 4 hr, the cells were washed twice with PBS and fresh hNSC media (without growth factors to stop proliferation) was added. The hNSCs were transfected on Day 0 and Day 2, and the gene expression were analyzed on Day 3 (for the Sox9 gene) and Day 5 (for the Tuj1 gene) through qPCR. To quantify cell viability, a MTS assay (Promega) was performed on Day 5. For the study involving patch-clamp, the cells were seeded on cover slips with media exchanges occurring every other day.

**Immunocytochemistry:** All fluorescence images were obtained using a Nikon T2500 inverted fluorescence microscope. To investigate the nuclear localization of the dye-labeled NanoScript in hNSCs, the media was removed and the cells were fixed for 15 minutes in formalin (Sigma) followed by two washes with PBS. The nucleus was stained with DAPI (Life Technologies) for 30 minutes and then washed with PBS three times.

To investigate the extent of neuronal differentiation on Day 5, the hNSCs were fixed with formalin for 15 minutes and then washed twice with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and non-specific binding was
blocked with 5% normal goat serum (NGS, Life Technologies) in PBS for 1 hr at room temperature. To study the extent of neuronal differentiation, the mouse monoclonal antibody against *Tuj1* (1:200 dilution, Covance MMS-435P) and a rabbit polyclonal antibody against *Sox9* (1:200 dilution, Abcam ab26414) 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 washing three times with PBS, the samples were incubated for 1 hr at room temperature in a solution of anti-mouse secondary antibody labeled with Alexa Flour 568 (1:100, Life Technologies) and DAPI (1:100, Life Technologies), in PBS containing 10% NGS, and washed three times thereafter.

**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 below.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer (5’ – 3’)</th>
<th>Reverse Primer (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuj1</td>
<td>GGCCTCAACCCCAAAATTCAGG</td>
<td>CAGCTCCGACAGATCCAGT</td>
</tr>
<tr>
<td>Sox9</td>
<td>ATCACCCGCTCAGTACGA</td>
<td>GTGGCTGTAGTAGGAGCTGG</td>
</tr>
<tr>
<td>GFP</td>
<td>CCACATGAGCGAGGGACTTT</td>
<td>GGTGCGCTCCTGGACGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CATGGTCCAATATGATTCCACC</td>
<td>GATGGGATTCCCCATGGATGAC</td>
</tr>
</tbody>
</table>

**SEM Imaging Preparation:** To investigate the morphology of neuronal differentiation, the basal medium of mature neurons was removed and the neurons were fixed for 15
minutes in Formalin solution (Sigma) followed by two PBS washes. The biological samples were then dehydrated in ethanol. The dehydration process entailed replacing PBS with 50% ethanol/water, 70% ethanol/water, 85% ethanol/water, 95% ethanol/water, and absolute ethanol for 10 minutes each in succession. The biological samples were then stored in absolute ethanol before transferring to critical point dryer to eliminate traces of ethanol. Then 20 nm of platinum was sputter coated onto the surface of biological samples after drying. Zeiss Sigma Field Emission-Scanning Electron Microscope (FE-SEM) was used to acquire the micrographs.

**Calcium Imaging:** Fluorescent calcium indicator dye Fluo4 AM (Life Technologies) was used for calcium imaging experiments. The dye was dissolved in DMSO and added to the cell culture to achieve a final concentration of 2 μM. Cells were incubated for 20 min with Fluo4 AM dye. Free dye was washed out with pre-warmed HBSS (Life Technologies). Cells then were incubated for 30 min in DMEM media (Life Technologies) for de-esterification of the Fluo4 AM dye. The media was replaced with pre-warmed HBSS during the imaging session. Images were acquired using a Zyla sCMOS camera (Andor) mounted on an Olympus IX71 using a 20x, 0.75 NA objective. Images were taken at 0.5 sec intervals for 1 min. Calcium imaging movies were displayed at 20 Hz. Images for calcium imaging were processed and intensity was quantified using the NIH ImageJ software.
**ICP-OES Measurement:** After hNSCs were seeded in a 12-well plate (125,000 cells/well), 1 nM of NanoScript was added. After 6 hours, the cells were washed with PBS and detached using Accutase (Gibco). After centrifugation, the supernatant was removed and 25 μL of lysis buffer was added. Then 180 μL 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 5.8 mL of distilled water (6 mL total volume with 3 % aqua regia). Cellular uptake experiments were performed three times and each replicate was measured for gold, iron, and zinc content three times by Inductively coupled plasma optical emission spectroscopy (Perkin Elmer Optima 7300 DV), operating under normal conditions.

**Cell Transmission Electron Microscopy (TEM):** hNSCs cells were cultured with NanoScripts using the same method as above. 24 hr post-transfection, the cells were trypsinized and fixed with Trump’s Fixative (Electron Microscopy Sciences) for 1 hr, washed with sodium cacodylate buffer (Electron Microscopy Sciences), suspended in a 1% osmium tetroxide solution for 1 hr, washed with water, and then progressively dehydrated with ethanol (50, 70, 80, 95, 100%). Then the cells were embedded in epoxy resin using the Low Viscosity Embedding Media Spurr’s Kit (Electron Microscopy Sciences) following the manufacturer’s protocol. The images were obtained with the JEOL 100CX TEM.
5.3 RESULTS AND DISCUSSION

5.3.1 Design of Repression-specific molecules for NanoScript

The NanoScript platform was functionalized with multiple molecules in order to emulate the function and structure TF repressor proteins. The first component of NanoScript is the hairpin polyamide molecule, specific for the GFP and Sox9 genes. The hairpin polyamide is a small molecule comprised of the pyrrole (Py) and imidazole (Im) groups which binds to A-T and G-C base pairs on the DNA respectively with nanomolar affinity.14, 15 The binding of the hairpin polyamide to the DNA sterically hinder the attachment of enzymes like RNA Polymerase II to the binding site, which in turn, prevents the gene from being transcribed.11 The GFP promoter sequence was obtained from the company from which GFP-labeled rat neural stem cells (rNSCs) were purchased (Figure 5.2). A hairpin polyamide with a sequence of PyPyPy-β-PyPyIm-γ-PyPyPy-β-PyImPy-β-

Figure 5.2: GFP Promoter Sequence. The GFP-labeled rat neural stem cells (rNSCs) were commercially purchased and the first 700 base pairs upstream from the GFP promoter region was provided by the vender. This GFP sequence contains several motifs (highlighted in green) which complement the 5’-CANNTG-3’ target sequence of the GFP polyamide.

GAAGAACACCACTCTTTCTGCCAGGAAGTGATGACGTACAACGCTTGGAGGGCAGCTCCATCGCTTGTGAAG
AACCACGTCTTTACCATGGAAGGCTGGCAGGCAAGGCAACTCTGTGTTCCGCAACCATAATTGTT
GCCAGATCCGGCTGACCGACGCCCCTCCCTGCTCCTCCGACATCGTGAACCGCGGCTCCGGCTGAG
TCCAGTGACGCAACCGGTACCTTCAGAAAGTACCCCAACCAATGACGCAACTACCTCCAGTTCC
AGCTCCCCTGGCTTCAATGACCCGCCACCTGCCCCTACAGGACGACGGCCCTGCTGGTGA
GATCAGCAGCAGACATCAAATCGAGAGCAAGATTTCTGTAACGCGTGGAGTCAAGGC
AGCAACTTCCCCGACAGCGCCCCTGATGCAAGACATCGTCCGCATCGACCCAGCT
TCGAGGCCATGTAATGAACAGGCAGCTGTGCCTGTGGCCAGGTCATTCTGTGGTAC
GACCACGGAACAGTACGACATCGAACCCCTGATGAAAGAACAGGGGTGGATC
AAGGAGTCCACGATACTCTATCCTCAAGCCACCGCCTGGAGAGACCTACGTGGAGAGACG
GGCGCTCGTGAGCAAGACGGACCGCCATCGCCCGAGATCGACCACGCGAGACCCCTGCGGATCATCGACGACTGCAGTGGTGAC
Dp-NH₂ (γ is γ-aminobutyric acid, β is β-alanine, and Dp is dimethylaminopropylamide) that targets the GFP promoter was synthesized using a previously established solid-phase synthesis protocol (Figure 5.3a). An in-vitro binding assay study was performed using surface plasmon resonance (SPR) and revealed a high nanomolar binding affinity (Figure 5.3b). Moreover, we synthesized a Sox9-specific hairpin polyamide with a sequence of

![Figure 5.3: Structure and Binding Affinity for GFP Polyamide.](image)

(a) The hairpin polyamide specific for GFP was synthesized to target the GFP promoter 5'-CANNTG-3', with the pyrrole group targeting the A-T base pairs and the imidazole group targeting the G-C base pairs. (b) Surface plasmon resonance (SPR) sensograms show the binding characteristics of the GFP polyamide to the target GFP sequence, and reveals a tight nanomolar binding affinity of 9.0×10⁻⁹. The equilibrium constant (KD), which is indicative of the binding affinity, was determined by the ratio of the dissociation constant (kd) to the association constant (ka).
PyPyPy-β-PyImPy-γ-PyPyPy-β-PyImIm-β-Dp-NH2 that also showed nanomolar binding affinity to its target sequence. (Figure 5.4).16

The second molecule is the corepressor peptide with a sequence of WRPW. The WRPW peptide was specifically chosen because: 1) it has been demonstrated to induce gene repression by preventing the formation of the basal transcriptional machinery at the binding site, 2) it induces repression of genes via the Groucho family proteins, which have

![Sox9 Polyamide](image)

**Figure 5.4: Structure and Binding Affinity for Sox9 Polyamide.** (a) The hairpin polyamide specific for Sox9 was synthesized to target the Sox9 promoter 5'-ACAATGG-3’, with the pyrrole group targeting the A-T base pairs and the imidazole group targeting the G-C base pairs. (b) SPR sensograms show the binding characteristics of the Sox9 polyamide to the target Sox9 sequence, and reveals a tight binding affinity of 5.4×10⁻⁸. The equilibrium constant ($K_D$), was determined by the ratio of the dissociation constant ($k_d$) to the association constant ($k_a$).
been demonstrated to play a role in neurogenesis, and 3) it is a short tetrapeptide with only 4 amino acids, and hence it is readily soluble in physiological environments.\textsuperscript{17-19} The third molecule is the membrane penetrating peptide (MPP) which has been previously demonstrated to effectively shuttle nanoparticles across the plasma and nuclear membrane.\textsuperscript{20}

5.3.2 Construction and Characterization of NanoScript for Gene Repression

These small molecules (hairpin polyamide, WRPW peptide, and MPP) were conjugated to PEG-based linker molecules to enhance their solubility, with the PEG linker having with a thiol terminus to enable functionalization onto the magnetic core-shell nanoparticles (MCNPs).\textsuperscript{21} MCNPs were chosen because of their high biocompatibility, inert properties, ability to induce magnetofection by placing a magnet underneath the culture plate to attract MCNP onto the cell surface, and multifunctional gold surface which enable attachment of multiple molecules on a single nanoparticle.\textsuperscript{22-24} After the nanoparticles were functionalized with the PEG-terminated small molecules (refer to methods in SI for details), the resulting platform was termed NanoScript (Figure 5.5a). Using a combination of dynamic light scattering and transmission electron microscopy, we found the size of the MCNP to be 17.3 nm (Figure 5.6), and after functionalization, the size of both NanoScript-GFP and NanoScript-Sox9 was found to be about 45 nm (Figure 5.5b). Through UV-vis absorption spectroscopy, we observed a shift in the plasmon
resonance which is indicative of surface functionalization (Figure 5.7). Based on previously demonstrated studies which show that there are approximately 4.3 ligands/nm², we predict that there are approximately 3,902 ligands on the nanoparticle. Moreover, the monodispersity of NanoScript was confirmed through transmission electron microscopy, wherein we visualized well-rounded and monodispersed sizes (Figure 5.5c). Furthermore, we tested if NanoScript can localize within the nucleus by labeling NanoScript with an
Figure 5.6: High Resolution TEM of MCNP. A high resolution TEM image of an MCNP reveals the total size to be 17.3 nm. Specifically, based on changes in the refractive shades between the gold (Au) shell and magnetic core, we approximated the magnetic core diameter to be 13.9 nm and the Au shell thickness to be 1.7 nm.

Figure 5.7: UV Absorbance of NanoScript. To confirm the functionalization of the magnetic core-shell nanoparticles (MCNPs), we performed UV absorbance. The unmodified gold-shell MCNPs showed a characteristic peak at 524 nm. Adsorption of the biomolecules alters the surface plasmon resonance AuNPs, and hence, we observed a shift in the absorbance peak of the NanoScript-GFP and NanoScript-Sox9 to 530 and 531 nm respectively.
Alexa Flour 568 dye and transfecting them into rat neural stem cells (rNSCs). After 24 hours, we performed fluorescence imaging and NanoScript was detected within the nucleus (Figure 5.5d, Figure 5.8). We also performed TEM on cellular cross-sections and found that NanoScript was distributed in the nucleus and cytoplasm (Figure 5.9). Moreover, we performed inductively coupled plasma optical emission spectrometry (ICP-OES) and observed that NanoScript was uptaken inside the cells (Figure 5.10).

![Figure 5.8: Intracellular Localization of MCNPs.](image)

**Figure 5.8: Intracellular Localization of MCNPs.** The intracellular localization of NanoScript was evaluated using fluorescence imaging. Results show that NanoScript (with the MMP and magnetic field application) is able to enter the nucleus. If the magnet is removed the nanoparticle (with the MMP) can moderately enter the cell due to the MPP peptide, but if the MPP is removed from the nanoparticle, also no particles enter the cell. (scale bar = 20 μm)

### 5.3.3 NanoScript for GFP Knockdown in rNSCs

To test if NanoScript can repress gene expression, we performed a proof-of-concept demonstration using a GFP-specific NanoScript (termed NanoScript-GFP) on GFP-labeled rNSCs. These GFP-labeled rNSCs intrinsically express GFP, and its expression can easily be detected through fluorescence imaging. Hence, we can evaluate the gene repression capability of NanoScript-GFP by observing GFP knockdown in rNSCs (Figure 5.11a). The
Figure 5.9: Cellular Cross-Section TEM. The NanoScript was transfected into the hNSCs, and after 24 hours, we performed TEM on the cellular cross sections. The NanoScript particles (yellow arrows) were found to be present in both the nucleus (purple outline) and the cytoplasm.

Figure 5.10: ICP-OES Quantification of MCNP Cellular Uptake. To evaluate the cellular uptake of MCNPs in hNSCs, we performed ICP-OES. After 6 hours of transfection, we observed that magnetic nanoparticles (MCNPs) are most uptaken when the MPP and magnetic field application is present. Without the magnet, about half the MCNP enter the cell, and if the MPP is removed, there is minimal uptake. The units in the vertical axis are nanograms of MCNPs uptaken per well (ng/well); each well contained 125,000 cells at the time of measurement.
rNSCs were transfected with NanoScript-GFP and expression of GFP was evaluated at different time points using fluorescence imaging. During the transfection, a magnet was placed underneath the culture plate for 15 min to induce magnetically-facilitated delivery, which is a technique to attract NanoScript onto the cellular surface. After 4 days, the GFP expression was significantly repressed as compared to the control; and control conditions which included unconjugated GFP polyamide, unconjugated WRPW peptide, nanoparticle with WRPW (MCNP-WRPW), and nanoparticle with GFP polyamide only (MCNP-GFP), showed decreased GFP knockdown (Figure 5.11b). In the control conditions, attachment

Figure 5.11: NanoScript-GFP Effectively Silences GFP Expression. (a) Schematic representation of NanoScript-GFP downregulating GFP expression (i.e. reduction in green fluorescence from the cell) in GFP-labeled rat NSCs. (b) Fluorescence images of GFP-labeled rNSCs 4 days post transfection showing a maximal decrease in GFP expression (green) when NanoScript-GFP is transfected (scale bar = 20 μm). A 1 nM concentration of MCNPs was applied during the transfection. (c) Quantification of GFP expression in the fluorescence images corroborates the trend in the images and reveals that NanoScript-GFP has the highest GFP knockdown as compared to the controls.
of either WRPW or GFP polyamide to the nanoparticle increased GFP knockdown as compared to unconjugated WRPW or GFP polyamide. The same nanoparticle core was used for each condition. Moreover, the MPP was shown to have almost no direct influence on GFP knockdown. The intensity of GFP fluorescence expression in the images of all the conditions was quantified, and these results not only confirmed the trend observed in the fluorescence images, but revealed a time-dependent increase of GFP knockdown. Finally, we tested GFP mRNA levels using qPCR and observed a similar trend of decreasing GFP expression using NanoScript. Collectively, these results suggest that NanoScript-GFP represses GFP expression and, the cooperative function of the polyamide and WRPW on the same MCNP enhances gene knockdown.
5.3.4 NanoScript Represses Sox9 for Neuronal Differentiation of hNSCs

Although this proof-of-concept demonstration indicates that NanoScript can repress gene expression, the real challenge and central goal is to translate the NanoScript-based gene repression approach for stem cell differentiation applications. To this end, the Sox9 gene has been identified as a critical gene to regulate neuronal differentiation in stem cells. Studies have shown that repression of Sox9 in neural stem cells initiates a pathway to guide their differentiation into neurons.\textsuperscript{26, 27} Hence, we developed a Sox9-specific NanoScript (termed NanoScript-Sox9), and we predict that if NanoScript-Sox9 can effectively repress Sox9 in human neural stem cells (hNSCs), enhanced differentiation into neurons can be observed (Figure 5.14a).
To test this, we transfected NanoScript-Sox9 into hNSCs (refer to methods in SI for protocol) and then we evaluated the expression of neuronal markers through qPCR and Figure 5.14: NanoScript-Sox9 Represses Sox9 to Induce Functional Neuronal Differentiation. (a) Schematic representation of Sox9 repression in human NSCs by NanoScript-Sox9 induces enhanced neuronal differentiation. (b) Fluorescence images of hNSC stained with Tuj1 5 days post-transfection shows greater Tuj1 expression (red) when NanoScript-Sox9 is transfected. (Scale bar = 20 μm). (c) Gene expression analysis using qPCR in hNSCs reveals that repression of Sox9 correlates with an upregulation of Tuj1. (Percent down-regulation of Sox9 and fold up-regulation of Tuj1 was calculated by normalizing to the housekeep gene, GAPDH, from the control) Standard error is from three independent trials (* = P < 0.05). (d) Spontaneous calcium fluctuations via Fluo4 fluorescence (orange/yellow color) for an active neuron (white circle) during 18 seconds of imaging (scale bar = 20 μm). (e) Traces for the normalized fluorescence change (ΔF/F0) representing spontaneous calcium ion influx for an active neuron (red line) and an inactive neuron (black line). Decreasing trend of the fluorescence is due to mild photobleaching.
immunocytochemistry. Specifically, the expression of *Tuj1* was evaluated because it is a prominent marker for neurons. We predict that the suppression of *Sox9* by NanoScript-Sox9 should lead to enhanced neuronal differentiation, and hence, an increase in *Tuj1* expression. We fixed and stained for *Tuj1* on Day 5, and the resulting fluorescence images indicated a greater expression of *Tuj1* as compared to the control (Figure 5.14b). This was further confirmed by testing gene expression through qPCR, wherein the expression levels induced by NanoScript-Sox9 showed a decrease of *Sox9* expression by 63% and a 5.7-fold increase in *Tuj1* expression as compared to the control (Figure 5.14c). The expression levels of other control conditions (nanoparticle with WPRW and nanoparticle with Sox9 polyamide) were also able to induce Sox9 repression and *Tuj1* expression, but not as strongly as compared to the NanoScript-Sox9 conditions (Figure 5.14c). The expression

![Graph showing gene expression](image)

**Figure 5.15: Gene Expression of Control Conditions.** To evaluate the effect of control conditions including Sox9 Polyamide only and WRPW Peptide only, we performed qPCR to measure the expression levels of *Sox9* and *Tuj1* at Day 3 and Day 5 respectively. Results showed that expression of Sox9 was minimally repressed and Tuj1 was minimally overexpressed. This trend follows previous literature wherein greater levels of Sox9 repression correlates to greater levels of Tuj1 overexpression. Expression levels were calculated by normalizing to the control and standard error is from three independent experiments.
levels of additional control experiments including unconjugated Sox9 polyamide and WRPW showed minimal changes as compared to the control (Figure 5.15). Expression of Sox9 protein levels was further evaluated using immunostaining which revealed a similar decreasing trend in the NanoScript-Sox9 condition (Figure 5.16). Moreover, by performing SEM, we were able to visualize neurons in high resolution (Figure 5.17). High cell survival was confirmed with a cell viability assay (Figure 5.18).

**Figure 5.16: Immunostaining of Sox9 Protein Expression.** The expression of Sox9 protein was evaluated via immunostaining and revealed a decreasing trend of Sox9 expression (green, right column) when the NanoScript-Sox9 was applied. (scale bar = 40 μm)
Figure 5.17: SEM of Induced Neuronal Morphology. We performed scanning electron microscopy (SEM) 5 days post-transfection to visualize the characteristic cellular morphology of the induced neurons (scale bar = 10 μm).

Figure 5.18: NanoScript has High Cell Viability. To evaluate the biocompatibility of our NanoScript conditions, we performed a cell viability assay (PrestoBlue) and found that all the conditions had a high viability as compared to the untreated control. Percent viability is relative to the control and standard error is from three independent experiments.
5.3.5 NanoScript-induced Neurons Show Functional Activity

To evaluate if the induced neurons have spontaneous neuronal activity, we monitored changes in intracellular calcium levels. Functionally active neurons are known to spontaneously fire action potentials that allow influx of cations including calcium.\(^{29}\) Using a commercially available calcium indicator dye, Fluo4, changes in intracellular calcium concentrations were visualized and its fluorescence intensity was quantified. After 7 days post-transfection, we performed calcium imaging using Fluo4 and observed changes in fluorescence levels in the induced neurons (Figure 5.14d). Furthermore, we quantified the fluorescence changes and observed spontaneous fluctuations of calcium ions in the active neuron over a 60 second period while the control inactive neuron did not show any changes in fluorescence (Figure 5.14e). These results suggest that the induced neurons show functional activity.

5.4 CONCLUSION

In summary, the overall goal of introducing a tunable and efficient platform that can mimic TF repressor proteins for effectively repressing genes to induce stem cell differentiation was achieved. As a result, this is the first-ever demonstration of utilizing a nanomaterial-based platform for emulating the function of TF repressor proteins to downregulate gene expression at the transcriptional level for inducing stem cell differentiation. We developed the NanoScript platform by functionalizing a nanoparticle
with multiple gene repression molecules such as gene-specific polyamides and the WRPW peptide. We first show a proof-of-concept demonstration that utilizes a GFP-specific NanoScript to knockdown GFP expression in GFP-labeled rNSCs. Then we show that a Sox9-specific NanoScript can repress Sox9 expression in hNSCs to initiate enhanced differentiation into functional neurons. The only difference between these two demonstrations is the gene-specific polyamide, thus highlighting the versatility and tunability of the NanoScript platform.

Furthermore, the results from both demonstrations (GFP knockdown and Sox9 repression) suggested that the synergistic effect of the polyamide and WRPW peptide on the NanoScript is needed for enhanced gene repression. One hypothesis for this result is that the two molecules contribute to gene repression through two different mechanisms. Previously reported mechanistic studies have shown that the binding of the polyamide to the target DNA sequence sterically occlude factors like RNA polymerase II for assembling on the DNA; and the WRPW peptide is known to initiate the Groucho family proteins which are well-established corepressor factors that prevents the formation of the transcriptional basal complex. By assembling both molecules on the NanoScript, we not only synergistically enhance gene repression, but enable NanoScript to more closely mimic the structure of TF repressor proteins.

While NanoScript does not completely knockdown GFP, its knockdown level is comparable to other nanomaterial-based methods that regulate GFP knockdown at the
translational level. Other methods for inducing neuronal differentiation such as viral vectors, small molecules, and nanomaterial-based platforms have been developed, but because of NanoScript unique features including its non-viral gene regulation and interchangeable components, we are further investigating to optimize and evaluate NanoScript against current methods. Furthermore, previous studies have shown that the extra cellular matrix plays a role in inducing neuronal differentiation, and so, the effect of ECM on assisting in NanoScript-based differentiation may require further investigation.

Thus far, the current NanoScript platform is primarily applicable for gene regulation in adherent cells, but when NanoScript was evaluated for gene regulation in hNSCs cultured in suspension, we observed knockdown of the Sox9 gene (Figure 5.19); however, we are further optimizing and investigating the potential of applying NanoScript for other cell types, such as those in suspension.

![Figure 5.19: NanoScript-based Gene Repression in Suspension Cells.](image)

The NanoScript-Sox9 platform was tested for its gene-regulating applicability in hNSCs suspension cells. The hNSCs were cultured in suspension and 3 days after NanoScript-Sox9 was transfected, we evaluated Sox9 expression levels. We found that NanoScript-Sox9 was able to knockdown Sox9.
In conclusion, the introduction of NanoScript platform as an approach to repress gene expression will significant impact the field of stem cell biology. First, because NanoScript regulates gene repression in a non-viral manner, it can be a candidate for stem cell-based research and potential therapies. Second, the high cell viability of NanoScript-transfected rat and human NSCs ensures the potential applicability of NanoScript for other stem cells lines. Third, by simply redesigning the polyamide sequence to complement a targeted gene, it is possibly to modify NanoScript to target and repress almost any gene of interest. We are confident that the versatility, effectiveness, and tunable properties of NanoScript will give scientists a new tool for gene-regulating applications such as stem cell biology and cellular reprogramming.

5.5 REFERENCES FOR CHAPTER 5


Chapter 6

Conclusions and Future Outlook

The NanoScript platform has been demonstrated as an effective tool for stem cell differentiation applications. Although some optimization is required, such as a more specific DBD, the tuneable platform can easily be applied for many other stem cell applications. NanoScript holds tremendous potential for commercialization and translation into the clinic.
6.1 SUMMARY & PERSPECTIVE

In summary, our major innovative idea was to develop a platform capable of replicating the exact functional structure and function of natural TF proteins (which are master regulators of gene expression and stem cell differentiation). NanoScript replicates the fundamental structure of TF proteins because the small molecules are tethered together in close proximity on a single gold nanoparticle. The gold nanoparticle is a functional component of the NanoScript because it serves as a backbone to integrate the individual small molecules onto a single construct. NanoScript is the first nanomaterial-based platform that mimics natural TFs and interacts with endogenous DNA to regulate gene expression at the transcriptional level in a non-viral manner.

NanoScript emulates the gene-regulating function of TFs because the four biomolecules on NanoScript, which include i) a NLS (Nuclear Localization Signal) peptide to shuttle NanoScript inside the nucleus, ii) a hairpin polyamide DBD (DNA Binding Domain) for binding to targeted DNA sequences, iii) a transactivation peptide AD (Activation Domain) for recruiting the RNA polymerase to the binding site to initiate transcription, and iv) epigenetic modification molecules to remodel the chromatin, all function synergistically to regulate transcriptional activity of targeted endogenous genes.

While all these components have been individually reported, our true innovation is integrating these individual components together onto a single platform for a functional
application of mimicking TF proteins for stem cell differentiation. NanoScript has several innovative and advantageous features: 1) it is inert and non-toxic, 2) gene regulation is non-viral, 3) the small molecules can be easily redesigned to target almost any differentiation-specific gene, 4) the ratio of small molecules on the gold nanoparticle can be easily tuned to enhance gene expression, and 5) the inert gold nanoparticle core (which is FDA-approved) which potentially allows for safe clinical translation.

The bio-inspired NanoScript platform was developed to replicate natural TF proteins for the goal of effectively regulating gene expression in stem cells in a non-viral manner. As a result, we can precisely control stem cell differentiation into specific lineages (i.e. muscle, cartilage, and neurons), which are clinical-grade and safe for animal/human transplantation. To this end, we have performed 4 demonstrations to validate that NanoScript can effectively and safely regulate gene in stem cells in induce their differentiation.

The first demonstration was a proof-of-concept study that introduced the NanoScript platform. We initially showed that NanoScript can effectively localize within the nucleus of the cells, which is important because transcription can occur only in the nucleus. Then, using a reporter plasmid system, we showed that NanoScript can effectively transcribe targeted genes in a dose-dependent manner. Furthermore, the multi-domain structure of NanoScript was validated to replicate the multi-domain structure of TF proteins. Finally, we observed that NanoScript is able to transcribe endogenous genes on native DNA.
The second demonstration focused on utilizing NanoScript for differentiating stem cells into muscle cells. The powerful NanoScript platform can be easily modified to target any gene of interest just by changing the sequence of one of the molecules. Upon modifying NanoScript to target muscle-specific genes, we show that NanoScript differentiates stem cells into long, striated, multi-nucleated muscle cells which express distinct myogenic genes. The induced muscle cells were determined to be mostly free of any physical or genetic footprints, and hence they are safe to be potentially translated into the clinic for stem cell-based therapies.

The third demonstration strived to enhance the gene-regulating potency of NanoScript by introducing a small molecule called CTB, which functions to increase transcriptional activity. By supplementing the NanoScript platform with CTB, we show significantly increased expression of genes. Moreover, this CTB-enhanced NanoScript, which was designed to target chondrogenic genes, revealed a drastic enhancement in differentiating stem cells into cartilage cells as compared to the original NanoScript platform.

Finally, the forth demonstration focused on redesigning NanoScript to repress targeted genes. For this purpose, we modified the small molecule components on NanoScript with repressor domains and showed that NanoScript can effectively repress desired genes. By modified NanoScript to repress the Sox9 gene, we observed over 90% knockdown of Sox9 in stem cells, thus inducing neuronal differentiation. Surprisingly, these induced neurons showed spontaneous and functional activity.
Overall, NanoScript has been demonstrated to both activate and deactivate targeted genes for stem cell-based applications. The tunable and interchangeable components allow NanoScript to be target any desired gene, and hence, it is a powerful research tool for scientists to use in their stem cell experiments. Moreover, because these stem cells are differentiated in a non-viral, footprint-free manner, NanoScript holds great potential for clinicians to use in stem cell-based therapies.

6.2 FUTURE OUTLOOK

6.2.1 NanoScript for Research Applications

The tunable and interchangeable biomolecules of NanoScript can easily be modified to either activate or deactivate any gene of interest. This powerful feature—of being able to upregulate or downregulate any desired gene, including genes involved in stem cell differentiation—makes NanoScript a powerful research tool for stem cell scientists. Stem cell biologists are currently seeking new methods and strategies to generate terminal cells (e.g. neurons, cardiomyocytes, cartilage, pancreatic tissue, etc.) in a rapid, safe, effective, and non-viral manner. Because the DBD component of NanoScript can easily be modified to target almost any differentiation-specific gene, NanoScript can induce generation of almost any cell lineage. Therefore, the research potential and impact will be immense.
Specifically, NanoScript is now being redesigned to target differentiation-specific genes such as Oct4 and Sox2 to generate induced pluripotent stem cells (iPSCs), and Nurr1 and Ascl1 to generate dopaminergic neurons. Moreover, the gene-repression NanoScript is also now being investigated for cancer application such as silencing survival-related genes in targeted cancer cells to induce their apoptosis. At the same time, investigating alternative approaches to replace the current hairpin polyamide with a more specific DBD is also underway, with an interesting candidate being triple-helix forming oligonucleotides because they can be specific for much longer DNA base pairs such as 22 base pairs.

The protocol to develop and synthesize the NanoScript platform for any desired gene is publically available to the scientific community, and hence, any research scientist or stem cell scientist can use NanoScript for their experiments. Enabling research scientists to independently use NanoScript for their experiments would ultimately push the field of stem cell biology one step closer towards developing an effective stem cell-based therapy.

### 6.2.2 NanoScript Commercialization

The NanoScript technology was heavily vetted through the NSF Innovation Corps training program, wherein we learned the steps required to commercialize NanoScript. Specifically, licensing the technology to established companies was determined to be most practical and feasible approach to make NanoScript commercially available in a relatively short timeframe (1-2 years). Through the NSF I-Corps program, we determined that our
target customer segment for NanoScript are stem cell biologists. We also learned that packaging NanoScript as a gene-specific kit preferred these scientists. Finally, we learned that by partnering with an established company and licensing NanoScript to them is the fastest route towards commercialization.

6.2.3 Potential Clinical Translation of NanoScript for Stem Cell Therapies

Because NanoScript induces stem cell differentiation in a non-viral and footprint-free manner, NanoScript has enormous potential in the field of regenerative medicine and can be applied for stem cell-based therapies or research field where selective gene regulation is desired. While stem cell therapies appear very promising, there is a critical gap that exists between our current knowledge and the practical application of stem cell therapies in the clinic.

Because NanoScript is effectively excreted after inducing stem cell differentiation the generated muscle cells are free of any foreign substances, which render them desirable for clinical therapies. Moreover, because NanoScript does not integrate within the DNA, there is low probability of undesired mutations. This directly influences clinicians, who are seeking newer and safer therapies, and patients, who are seeking a potential treatment plan for debilitating diseases.

Specifically, there are two approaches for clinically-translating NanoScript: the first is transplanting NanoScript-induced cellular lineages (i.e. neurons, muscle, cartilage, etc.)
into in vivo models to restoration of lost behavior and function, and the second is directly injecting NanoScript into the injured/damaged site for in vivo cellular reprogramming. Although the latter will require significant more modification and optimization, the former approach is feasible for immediate evaluation in animal models. The clinical-grade cellular lineages induced by NanoScript hold significant potential to revolutionize the field of nanotechnology-based regenerative medicine.