RICE UNIVERSITY

INVESTIGATING OPTOGENETICS AS AN APPROACH TO CONTROL GROWTH FACTOR EXPRESSION FOR TISSUE ENGINEERING

by

Trenton Piepergerdes

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE IN BIOENGINEERING

APPROVED, THESIS COMMITTEE

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HOUSTON, TEXAS
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1. Abstract

The body exhibits a robust capacity for regeneration when faced with tissue injury or damage. In some cases, however, these insults can exceed the innate capacity for healing, resulting in permanent loss of structure and function. It is in these injuries where tissue engineering seeks to design interventions that can restore structure and function through the implementation of scaffolds, bioactive factors, and/or cells. Bioactive proteins have demonstrated immense efficacy in inducing tissue formation, but the administration of these factors has seen limitations that prevent them from seeing clinical success. Namely, precise spatiotemporal delivery of these factors is critical to their function and has yet to be achieved through exogenous delivery methods. **There is thus a need for technologies that enable precise spatiotemporal administration of growth factors for tissue engineering.**

To this end, we propose that the precisely tunable variables associated with light make it an ideal stimulus for growth factor administration. Specifically, we sought to explore two previously developed light responsive systems as tools for controllable growth factor expression in mammalian cells with our overall goal being to make a case for optogenetic tools for tissue engineering applications. First, we explored the functionality of a red light-inducible adeno-associated virus (AAV). We next investigated a near infrared (NIR) optically responsive transcription control system as tool for tuned growth factor delivery. Finally, we ran preliminary studies to explore the feasibility of working with optogenetic systems in three dimensions (3D) through characterization of the relationship between scaffold fabrication parameters and light absorbance. In all, we validated tools for optogenetic control of growth factor expression and demonstrated feasibility of the technique for growth factor delivery in tissue engineering.
2. Acknowledgements
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They say a calm sea does not a skilled sailor make, and I agree completely. While I would not wish to experience these three years over again, I am so thankful for the man they have made me. I am beyond excited to turn a new page and achieve my full potential in an environment where I have confidence in myself and am encouraged to lead with kindness, love, and joy.
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6. **Specific Aims**

In morphogenesis and innate injury repair, tissue formation relies upon the complex expression of signaling proteins by surrounding cells. In natural processes, cells are stimulated to produce these growth factors, which in turn stimulate phenotypic effects in surrounding cells through autocrine, paracrine, and endocrine signaling mechanisms. Specifically, growth factors and cytokines are responsible for directing stem cell differentiation and proliferation of local cells, recruiting other necessary cells, and stimulating deposition of tissue specific extracellular matrix (ECM). Efforts to mimic these properties with exogenous protein delivery, however, fall short of native protein expression. **A collection of tools for the precise spatiotemporal control over growth factor presentation to guide tissue formation is of immense importance to the field of tissue engineering.**

To this end, we propose herein to implement recently developed optogenetic systems. Light responsive proteins identified across prokaryotic and eukaryotic species demonstrate precise, orthogonal, and tunable behaviors when stimulated with certain patterns and wavelengths of light ranging from ultraviolet (UV) to infrared (IR). The goal of this work is to explore the feasibility of optogenetics as an approach for growth factor delivery in bone tissue engineering. To investigate this, we have devised the following specific aims:

*Specific Aim 1:* Demonstrate functionality of red light-induced adeno-associated virus (VNP-PIF6) as a tool for tuned growth factor delivery

*Specific Aim 2:* Demonstrate functionality of bathybacteriophytochrome-based transcription control systems for tuned growth factor delivery
Specific Aim 3: Characterize how different scaffold fabrication parameters impact the absorbance of light.

7. Background

7.1. The Paradigm of Tissue Engineering Informs Therapeutic Strategies

Tissue engineering seeks to facilitate tissue regeneration in cases where injury exceeds the scope of natural restorative mechanisms. Traumatic injury, tumor resection, and birth defects can result in complex defects that cannot heal on their own.\(^4\) Often, grafting serves as the gold standard for treating these so-called “critical defects,” however, this technique is limited by the amount of source tissue, donor site morbidity, and graft rejection in the case of allografts or xenografts.\(^5\) Strategies to augment the body’s capacity for healing typically rely upon scaffolds, bioactive factors, and/or cells, otherwise known as the paradigm of tissue engineering (Figure 1). Either individually or as composite interventions, these components serve to induce and guide tissue formation. Scaffolds provide structural support to the forming tissue and can be used independently or as carriers for the other two pillars of the paradigm of tissue engineering, bioactive factors and/or cells. Bioactive factors provide potent cues for the stimulation of tissue-specific phenotypes of receiving cells, be they endogenous or exogenous. The delivery of exogenous cells affords dynamic machinery evolutionarily suited for tissue formation and cell signaling.

Scaffolds are necessary components of interventions that require structural integrity (i.e., geometrically complex, load-bearing, etc.) but are often lacking in biological activity.\(^6\) This shortcoming is often overcome through multiplexing scaffolds with biological signals. Proteins and functional peptides have long been popular candidates for inducing tissue-specific cellular phenotypes. Decades of \textit{in vitro} work have produced candidates such as
bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF) that robustly induce osteogenic and vasculogenic/angiogenic cellular behavior, respectively. Due to specialization in the Mikos lab for osteogenesis and vascularization, BMP-2 and VEGF have been selected as model growth factors for this work. When these molecules are transferred to \textit{in vivo} models, however, the field increasingly finds complications related to molecular bioactivity, spatial patterning, and temporal dynamics to name a few. It is therefore a primary goal of the field to optimize the presentation of bioactive molecules to achieve improved tissue formation along with decreased negative effects.

![Diagram of tissue engineering]

Figure 1 \textbf{The Paradigm of Tissue Engineering}. Scaffolds, cells, and bioactive factors serve as the main constituents of tissue engineering interventions. Efforts to improve generation of functional tissues rely upon these components for support, cell signaling, and active remodeling of injury site.

\subsection*{7.2. BMP-2 and VEGF Structure and Function}

The work presented in this thesis is oriented around developing tools for growth factor delivery, specifically for bone tissue engineering. While myriad growth factors have shown
efficacy in critical bone defects, the most well characterized and frequently used are BMP-2 and VEGF; the former is a potent inducer of osteogenesis and the latter a key player in vascularization. BMP-2 is a member of the transforming growth factor-β family that is secreted and binds to cell surface receptors in many cells including mesenchymal stem cells, osteoblasts, and more. Upon receptor binding, BMP-2 induces osteogenic differentiation of stem cells, mineralization of ECM, and acts as a positive feedback loop in that it induces expression of other BMPs. Osteogenesis is incomplete, however, without coupling with the formation of blood vessels, as bone is a highly vascular tissue. Therefore, VEGF is a necessary component of complete bone formation. This molecule has demonstrated to be a rate-limiting factor of tissue vascularization. While VEGF has many isoforms, of particular interest for bone formation is VEGFA, which itself has multiple more isoforms as a product of alternative splicing. The products of this alternative splicing exhibit differential presentation patterns ranging from fully secreted (VEGF_{121}, the smallest functional isoform) to fully sequestered by the membrane (VEGF_{189}, the largest functional isoform) due to the presence or loss of two heparin binding domains near the C-termini. An intermediate isoform, VEGF_{165}, has been found to be both secreted and sequestered and consequently demonstrates the greatest *in vivo* vascularization as compared to the other isoforms. VEGF binds to VEGF receptors VEGFR1 and VEGFR2 on the surface of endothelial cells. This binding induces endothelial cell migration and tubulogenesis. In addition to their individual relevance, it has been demonstrated that VEGF and BMP-2 exhibit unique and critical feedback to one another during bone fracture healing.

7.3. Growth Factor Expression During Tissue Formation

It is first important to understand how different bioactive molecules, particularly growth
factors, are expressed in natural cell signaling processes. A wide variety of input signals are multiplexed by cell receptors. These signals are then distributed to different locations in the cell to initiate numerous canonical signal transduction pathways. Most commonly in tissue formation, these input signals induce changes in cellular protein expression profiles. Cells transduce signals through cooperative regulation of transcription, resulting in complex proteomic profiles. Figures 2 shows empirically determined protein expression profiles over the course of bone fracture healing. From these data, it is clear that each growth factor exhibits complex dynamics and that expression profiles between growth factors vary

![Figure 2 Morphogenic and Angiogenic Factor Expression During Fracture Repair](image)

Figure 2 **Morphogenic and Angiogenic Factor Expression During Fracture Repair.** Bone fracture repair depends upon the synergistic and complex expression of tens of genes responsible for tissue resorption, cell signaling, ECM formation, and more. *Adapted with permission from* (Gerstenfeld, L.C., Cullinane, D.M., Barnes, G.L., Graves, D.T., & Einhorn, T.A. (2003). Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation, *Journal of Cellular Biochemistry*). *Copyright (2018) John Wiley and Sons*
significantly in magnitude and over time. Complications arise further when the spatial patterning of the growth factor expression is taken into account, for the precise spatial presentation of growth factors is critical to their function.\textsuperscript{20-23} As an example, endothelial cells (ECs) migrate along a VEGF gradient \textit{in vivo}.\textsuperscript{24} We see specifically that BMP-2 exhibits elevated early expression then tapers to a medial state for the entire course of fracture repair. VEGF, however, seems to be displayed later and to a lesser degree for a shorter amount of time. Both growth factors also depend highly on the proper expression profiles of tens of other morphogens, enzymes, cytokines, and more to achieve proper osteogenesis and vasculogenesis.\textsuperscript{25,26} In all, native tissue formation relies upon unique expression profiles of numerous growth factors to optimally form tissue.

\textbf{7.4. BMP-2 and VEGF Delivery for Tissue Engineering}

Taking advantage of the signal induction of growth factors \textit{in vivo} turns out to be a non-trivial task. Proteins must maintain bioactivity upon implantation into defect sites and this bioactivity must be at concentrations high enough to induce a therapeutic response but not so high that side effects become a concern. Specifically, supraphysiological concentrations of many potent growth factors can induce inflammation and even tumorigenic behavior in endogenous or co-delivered cells.\textsuperscript{27} Precise localization of growth factor presentation to the defect site is critical to its function. For example, diffusion of delivered growth factors or migration of therapeutic cells away from the defect site can result in off-target tissue growth.\textsuperscript{28,29} In addition to spatial fidelity, maintaining this therapeutically relevant concentration must occur over timescales relevant to tissue formation which is often on the order of weeks to months (Figure 2).\textsuperscript{30} To this end, the development of polymeric systems to deliver growth factors in a spatially and temporally controlled manner has dominated the
field of tissue engineering. Other work has explored achieving the same ends via gene therapy or the delivery of engineered cells.

7.4.1. Materials-Based Delivery of BMP-2 and/or VEGF

Early efforts to implement bioactive cues in tissue engineering interventions employed purified whole proteins. A bolus injection of VEGF into models of hind limb ischemia demonstrate improved revascularization as compared to sham controls. In most cases, however, growth factors must be administered in a more controlled fashion over space and time. Polymeric scaffolds have been investigated as tools to carry whole protein payloads while defining and maintaining the defect space. Proteins can be incorporated into the 3D scaffold non-covalently (surface adsorption, affinity binding, physical encapsulation, etc.) or covalently via chemical conjugation. In any case, different methods are chosen based upon the desired spatial and temporal dose profile of the delivered growth factor. In this section, we will focus on various carrier designs for VEGF and BMP-2 monotherapy or co-delivery for vascularized bone defects.

7.4.2. Single Protein Delivery

BMP-2 is a potent inducer of osteogenic phenotypes, thus necessitating precisely administered doses to avoid bone overgrowth, ectopic bone growth, inflammation, or even tumorigenic potential. Empirical evidence has suggested that, as opposed to a bolus/burst release, a steady, long-term release tends to produce better outcomes. Therefore, scaffolds to deliver BMP-2 are selected based upon their ability to prolong growth factor release and retention. Ehlert et al. employed a method to covalently attach BMP-2 to silica surfaces via amino-silane reactions, thus immobilizing the growth factor and potentially increasing the time scale of its presentation.
modified to slow BMP-2 release, such as vitrifying collagen I fibers.\textsuperscript{38} The vitrified scaffold released three-times less BMP-2 at 14 days as compared to unvitrified collagen I scaffolds, and subsequently induced enhanced osteocalcin expression and calcium deposition at 4 weeks. The interactions between BMP-2 and collagen can be improved by adding an N-terminal collagen binding domain, thus extending release at 12 weeks as compared to unmodified BMP-2.\textsuperscript{39} Srouji et al. achieved significantly delayed and extended BMP-2 release via poly(ε-caprolactone) (PCL) and poly(ethylene glycol) (PEG) electrospun meshes.\textsuperscript{40} With this method, only 15% of loaded BMP-2 was released after 27 days. At best, methods to deliver BMP-2 have achieved slow, linear release profiles that mitigate negative side effects but do not closely mimic the natural BMP-2 expression shown in Figure 2.

Explorations into delivery of VEGF for bone fracture healing has indicated similar patterns in that the prolonged presence of VEGF signaling can improve neovascularization of the newly formed bone.\textsuperscript{41} Complicating this, excessive delivery of VEGF can lead to leaky vasculature which in turn may cause edema and hypotension.\textsuperscript{42,43} De la Riva et al. achieved extended release of VEGF through encapsulating VEGF proteins in alginate microparticles and administering them on alginate/chitosan/poly(D,L-lactic acid) (PLA)-H release systems that provided physiologically relevant VEGF concentrations over 5 weeks.\textsuperscript{44} Another study encapsulated VEGF into PLA scaffolds and saw steady release over 4 weeks.\textsuperscript{45} In both cases, VEGF improved blood flow to the newly formed bone and thus improved bone formation. That said, VEGF plays a critical but auxiliary role in bone formation and is thus rarely used as a monotherapy for bone defects. Most often, VEGF acts to complement and enhance BMP-2-induced bone formation.
7.4.3. **Dual Protein Strategies**

Bone repair relies upon the orchestrated expression of multiple growth factors concurrently.\(^{29}\) Therefore, groups have employed many of the aforementioned strategies to achieve dual growth factor release, particularly with BMP-2 and VEGF due to extensive demonstrations of their synergy.\(^{46}\) Early work explored delivering the two growth factors simultaneously. Patel et al. implemented gelatin microparticles laden with either BMP-2, VEGF, or a combination of the two.\(^{16}\) These microparticles were further supported structurally within a poly(propylene fumarate) (PPF) scaffold and implanted into a critical size cranial defect. Results from histology and micro-computed tomography indicated some minor advantages to the combination group at four weeks, but those differences were minimal after twelve weeks. The group hypothesized that their inability to tune active growth factor concentration and the lack of spatial precision in said delivery prevented them from seeing the full effect of combinatorial work. A second paper was published where they explored multiple concentrations of BMP-2 and VEGF in the same scaffolds described above.\(^{47}\) Here, they found significant differences between various BMP-2:VEGF ratios, suggesting that the magnitude and ratio of presented growth factors are critical for bone formation. Reyes et al. confirmed this result with a similar study observing how the BMP-2:VEGF ratio impacts bone formation in a rat intermedullary femur defect when released from porous poly(lactic-co-glycolic acid) (PLGA) scaffolds.\(^{48}\) Results similarly suggested that the advantages apparent at early timepoints diminished after 12 weeks. In this case, the authors directly called for work to improve the overall dosing and timing of the two growth factors. Further, strategies implementing gene activated matrices (GAMs) have demonstrated the ability to achieve BMP-2 and VEGF
transgene expression via synthetic nano-hydroxyapatite polyplexes. These efforts too referenced a limited ability to tune the dose of each factor.

A common pitfall to the BMP-2 monotherapies or simultaneous dual delivery of BMP-2 and VEGF is that the osteogenic potency yields bone growth before sufficient vascularization occurs, often resulting in necrosis of forming tissue. To address this, groups have explored the sequential delivery of VEGF followed by BMP-2 with the goal being to delay calcification enough that a vascular network can take hold. Kanczler et al. achieved the sequential delivery through differential degradation properties of the polymers selected for VEGF and BMP-2 delivery. Specifically, an alginate hydrogel loaded with VEGF degraded more quickly than a PLA scaffold resulting in a rapid VEGF release profile as compared to BMP-2. Another group encapsulated BMP-2 into PLGA, which was then coated onto an allograft scaffold. Once coated with BMP-2-PLGA, VEGF was allowed to adsorb onto the surface, resulting in early VEGF delivery followed by BMP-2 from the slowly degrading PLGA coating. A similar approach took advantage of a rapidly dissolving VEGF-laden hydrogel surrounding a BMP-2-laden PPF rod to induce sequential delivery. In all, sequential delivery demonstrated improvements over BMP-2 delivered alone across many variables such as blood vessel density and bone volume. While each method yielded promising results, none compared the sequential delivery to dual delivery thus making direct comparisons difficult.

Delivery of whole proteins is a powerful method for induction of tissue formation; however, scaffold-dependent release methods lack the spatial and temporal precision to guide tissue formation to a functional output without potentially dangerous side effects. Further, the finite loading capacity of these approaches limits the timescale of release,
often leading to insufficient vascularization and tissue formation. Growth factors delivered within 0 to 3 days of injury will fall within the inflammation window of fracture healing and will thus be subjected to neutrophils, macrophages, and hypoxia, significantly decreasing their half-life.\textsuperscript{4,30} This necessitates the delivery of supraphysiological concentrations of therapeutic proteins, which exacerbates negative side effects. Beyond this, the hypothesized benefits to multiplexing of BMP-2 and VEGF have not been consistently demonstrated. In the described cases, groups attributed this to a lack of spatiotemporal control and lack of the ability to test a wide range of doses and ratios in a precise way.

7.4.4. **Cell-based Administration of BMP-2 and VEGF**

Cells provide several advantages that cannot be achieved in protein delivery alone. First, cells demonstrate theoretically infinite growth factor expression, limited only by the metabolic resources available and the lifetime of the cell.\textsuperscript{52} Second, cells are able to sense and respond to their environment, suggesting that they will be able to achieve optimal release profiles and concentrations for the needs of the defect.\textsuperscript{53} Third, cells are capable of synthesizing any protein encoded in their genome or episomally, thus making them delivery vehicles for all growth factors as compared to the delivery of single or dual growth factors in scaffold-based methods. Finally, the use of multipotent stem cells confers the advantages of multiple cell types as the cells differentiate.\textsuperscript{54} This section will discuss methods employing either direct modification of native cells or the delivery of cells engineered \textit{ex vivo/in vitro} to induce expression of BMP-2 and/or VEGF.\textsuperscript{55}

7.4.5. **In Vivo Gene Therapy**

Native cells are recruited to injured tissue and are responsible for signaling cascades that
induce tissue regeneration. Where growth factors fail, gene therapy approaches allow for the modification of these native cells *in situ* in order to achieve desired gene expression behaviors. For this reason, the use of cells modified to overexpress BMP-2 or VEGF has become an attractive technique to ensure therapeutic growth factor expression. Here, we will discuss methods for inducing BMP-2 and/or VEGF overexpression at the defect site.

7.4.5.1. Viral Vectors

Viruses have an innate proclivity for infecting mammalian cells with their genome. In this way, the host cells manufacture the proteins required for additional virus synthesis. Scientists have taken advantage of this unique characteristic by replacing viral genomes with transgenes of interest which are then packaged into viruses produced by capsid proteins transfected on a plasmid in *trans*.

These viruses can then be harvested and used to infect cells with the packaged transgene only, resulting in transgene expression and no additional virus production. A popular candidate for viral transduction of therapeutic genes is adeno-associated virus (AAV) due to its demonstrated safety profile, high transduction rates in multiple mammalian cells lines, and prolonged transgene expression. Gafni et al. implemented an AAV delivery system to transduce cells within a defect with doxycycline (Dox)-inducible BMP-2. Preliminary results indicated that the +Dox mice saw increased bone formation as compared to –Dox groups, suggesting therapeutic levels of BMP-2 were induced. A second common viral vector, adenovirus, has been employed to transduce local cells to express BMP-2 via direct injection into defect sites by Eggermann et al. This method yielded quantifiable BMP-2 expression upwards of 6 weeks following injection. The use of adenovirus did yield an increased immune response that limited the formation of viable bone tissue in
the defects, a characteristic that limits its clinical use.\textsuperscript{60}

Viruses can also be employed as components of GAMs that act as carriers for viral nanoparticles. GAMs are physical structures that allow for the delivery of plasmid DNA to induce transgene expression in local cells.\textsuperscript{61} Freeze-dried AAV delivering VEGF and receptor activator of nuclear factor κB ligand was coated on the surface of an autograft implant and administered to a mouse femoral defect.\textsuperscript{62} This therapy resulted in improved bone volume and vascularization as compared to the delivery of an innocuous β-galactosidase gene. A similar study employed the delivery of VEGF\textsubscript{121} in an attempt to improve bone formation and vascularization in a rabbit model of femur head necrosis.\textsuperscript{63} This approach increased the number of new blood vessels, improved bone density, and improved trabecular morphology over 12 weeks as compared to untreated controls. Liu et al. delivered adenovirus containing BMP-2 on a GAM in a cranial defect.\textsuperscript{64} Here, they determined that adenoviral transduction of local and delivered cells with BMP-2 yielded greater bone volume and bony bridging as compared to unmodified groups. BMP-2 and VEGF were multiplexed in a study that employed adenoviral delivery of both BMP-2 and VEGF, or adenoviral delivery of one and protein delivery of the other.\textsuperscript{65} Results indicated that early “burst release” of whole protein VEGF followed by a delayed and increasing release of BMP-2 through adenoviral transduction yielded optimal vascularized bone formation in a dental defect when compared to the other combinations.

\textbf{7.4.5.2. Synthetic Vectors}

Non-viral gene therapy offer distinct advantages in their limited immunogenicity, increased cargo capacity, and simplicity of fabrication. Cationic synthetic polymers
complex with plasmid DNA to create polyplexes which can then be incorporated into implanted scaffolds. With this method, Wegman et al. demonstrated successful transfection of local cells with either naked plasmid encoding BMP-2 or Lipofectamine/BMP-2 polyplexes. While both demonstrated improved osteogenesis, the polyplexes saw a greater induction of BMP-2 expression and subsequent osteogenesis, most likely due to its increased transfection efficiency over naked DNA. Another group explored cationized gelatin microparticles as a way to deliver BMP-2 plasmid DNA on oligo(poly(ethylene glycol) fumarate) (OPF). This prolonged DNA bioavailability and subsequent BMP-2 expression. Cumulative release of BMP-2 was also achieved with branched polycationic polymers by Chew et al. This release, however, did not yield improvements in a calvarial defect model as compared to no-plasmid controls. This suggests that the transfection efficiency may be too low to achieve therapeutic levels of BMP-2. One study incorporated plasmid-VEGF into collagen sponges and implanted into radial defects. This yielded improved osteogenesis and angiogenesis as compared to raw VEGF protein delivery; however, no comparison was made to BMP-2 delivery.

Gene therapy approaches for tissue engineering see many limitations when administered in vivo. First, transfection or transduction efficiencies are significantly lower than in in vitro experiments, making it difficult to reach therapeutic transgene concentrations. This necessitates the delivery of immense quantities of the selected carrier, resulting in increased risk for immune response. Further, localization of the vector to a specific injury site is non-trivial as most vectors are diffusible through the tissues and capillary membranes and display unique biodistributions. This results in transgene delivery to off-
target sites, particularly to the liver and kidney as the vectors are filtered from the circulatory system. Transgene expression from plasmids is highly context dependent and very few groups have investigated the effects of DNA level changes (promoter strength, genetic insulators, location in the genome if integrated, etc.). Finally, most vectors induce transient expression due to episomal infection. This is suboptimal for tissue engineering as growth factors are expressed over the entire time course of tissue regeneration which can last upwards of months.

To address the weaknesses associated with *in vivo* gene therapy, many groups have developed technologies and protocols that allow for the exogenous manipulation of cells and their subsequent implantation into defect sites. Cells, either allograft or autograft, are cultured *in vitro* and the selected transfection/transduction protocol is carried out. Successfully edited cells are then selected (via fluorescent reporters or antibiotic resistances included in the transgene) and grown up to reach sufficient numbers for implantation. Finally, the population of edited cells is then implanted into the defect as “factories” of the protein of choice. This allows the circumvention of immune response to administered gene therapy vectors.

A major limitation of cell therapies is the migration and death of administered cells, so achieving sufficient transgene expression is critical and often difficult.

### 7.4.6. Conclusions

The wide variety of bone injuries (geometry, mechanical requirements, etc.) require a variety of BMP-2 and VEGF release kinetics. To this end, both scaffold-based and gene therapy-based approaches are limited in their ability to provide user controls over spatial
and temporal tunability. The field of tissue engineering is in need of tools and protocols to achieve this specificity in order to achieve clinical promise.

7.5. Optogenetics

While promising, all previously discussed methods are lacking in their ability to afford clinicians control over the spatial and temporal dosing of growth factors. We hypothesize that optogenetics may serve as a novel way to control growth factor expression. Recent efforts have identified and employed many light responsive proteins from mammals, plants, and bacteria. Light is an attractive stimulus because it is orthogonal with mammalian cellular signaling networks (apart from the eye), it can be delivered with immense spatial and temporal precision, and it can penetrate the skin with limited cytotoxicity in the visible spectrum. Beyond this, light induced interactions are usually reversible, thus affording dynamic function to tools based upon these proteins. Many proteins have been investigated that exhibit complex responses to light, including conformational changes, alteration of membrane potential, homodimerization, heterodimerization, and clustering, among others. Looking toward a practical optogenetic tool for use in tissue engineering, an ideal system would have the following characteristics: (i) respond to light within the penetration window of human tissues (>650 nm), (ii) function properly in mammalian cells, (iii) tune response as a function of activating/deactivating light intensity, (iv) exhibit a reversible interaction, and (v) maintain functionality on timescales relevant to growth factor expression during tissue formation.

7.5.1. Tools for Optogenetic Control of Gene Expression

7.5.1.1. GI-FKF1

The dawn of optogenetic systems relied upon the dimerization of two distinct proteins in
response to light to achieve myriad impacts on cellular behavior. A common goal among tissue engineers and beyond is the ability to control gene expression. With this as inspiration, many groups have engineered these proteins to act as transcription control systems. An early group investigated two proteins from *Arabidopsis thaliana*: GIGANTEA (GI) and the light-oxygen-voltage (LOV) domain-containing FKF1, two proteins that heterodimerize in the presence of the chromophore flavin mononucleotide (FMN) in response to 450 nm light. The group capitalizes upon this functionality by fusing a DNA binding domain (Gal4) to GI and a transcription activating domain (VP16) to FKF1 and incorporating a luminescent reporter protein under control of a Gal4 inducible promoter (P$_{UAS}$). Under dark conditions, GI-Gal4 and FKF1-VP16 do not associate and luciferase is weakly induced due to leaky transcription from the promoter. Upon irradiation with blue light, however, GI-Gal4 and FKF1-VP16 heterodimerize and induce transcription up to five-fold higher than the off state. While promising, the blue light used to activate this system exhibits minimal penetration depths and significant cytotoxicity through DNA strand breaks.

7.5.1.2. CRY2-CIB1

Of particular import to this thesis, a group recently developed an optogenetic system with a goal to control growth factor expression. The approach employed by Polstein *et al.* relies not upon the uniting of a DNA binding domain and transcription activation domain as prior groups have, but upon the reconstitution of a Cre recombinase in response to blue light. Briefly, the Cre recombinase was divided into two parts and each part was attached to one component of the CRY2-CIB1 heterodimerizing protein pair from *A. thaliana*. First, a construct was designed that placed a gene of interest
(GOI) downstream of a constitutive promoter; however, an expression cassette flanked by LoxP recombinase sites was placed in between the promoter and the GOI, thus inhibiting expression of said GOI. Upon illumination with 450 nm light emitting diodes (LEDs), the two components of the recombinase were reconstituted and the inhibitory expression cassette was removed. This induced expression of the GOI, resulting in a permanent “on” switch. In this way, Polstein et al. showed guided differentiation of multipotent mesenchymal stem cells (MSCs) down a myogenic lineage when the GOI was MyoD, a master transcription factor for myogenesis. Further, the group demonstrated light-induced formation of blood vessels \textit{in vivo} when the GOI was VEGF and angiopoietin-1 fused with a self-cleaving P2A peptide. This is the first demonstration of the applicability of optogenetic systems for tissue engineering applications.

While promising, this system does display critical weaknesses: first, it is responsive to 450 nm light which necessitated the use of a transparent window for \textit{in vivo} application, thus limiting its therapeutic applicability; second, the system is a permanent “on” switch, which limits the user’s ability to achieve complex dynamic expression profiles that actually occur during tissue formation (Figure 2); and third, the \textit{in vivo} model was a poor proxy for actual use in tissue engineering as it employed HeLa cells, an immortalized cancer cell line fully incompatible for transplantation.

\textbf{7.5.1.3. PhyB-PIF6}

Groups have looked for red-shifted proteins in order to limit cytotoxicity and achieve greater tissue penetration. A plant phytochrome, Phytochrome B (PhyB), and phytochrome interacting factor 6 (PIF6) from the same species as the GI-FKF1 system,
*A. thaliana*, shows interactions amenable to optogenetic systems (Figure 3a).88 This group took a similar approach as the GI-FKF1 system and attached a DNA binding domain (TetR) to PIF6 and a transcription activating domain (VP16) to PhyB in order to induce transcription upon dimerization of PhyB-VP16 and PIF6-TetR. Light responsivity is conferred to the system by the chromophore phycocyanobilin (PCB), a reversible chromophore that alternates states in response to red (660 nm) and far-red (740 nm) light.89 With this system, researchers demonstrated up to 65-fold induction by red light of reporter expression, tunability of reporter expression in response to activating red light intensities, and spatial patterning of fluorescent reporter expression with photomasks.88 While this effort demonstrated use of a longer wavelength and a much larger dynamic range, the chromophore PCB is not endogenous to mammalian cells and must be added either exogenously or on the transfected/transduced plasmid.90 The addition of diffusible exogenous chromophore could lead to off-target effects and limits the system with a finite amount of chromophore. Expression of the chromophore from a plasmid improves the amount available for light induction but significantly increases the size of the delivered plasmid and the metabolic load placed upon the treated cells.

### 7.5.1.4. VNP-PIF6

Novel work in the Suh Laboratory at Rice University implemented the heterodimerizing functionality of the PhyB and PIF6 proteins to create a light-inducible AAV nanoparticle for spatially controlled transduction. AAV is a promising candidate for viral gene therapy as it is non-pathogenic, reasonably potent in mammalian cells, yields transgene expression on the order of months, and is minimally immunogenic as compared to other
viral methods. The AAV genome contains only two genes making it simple to manipulate at the DNA level. AAV is structurally comprised of three capsid proteins (VP1, VP2, and VP3), the first two of which have demonstrated to be amenable to modifications and amino acid insertions while maintaining overall structure and transducibility of the virus (Figure 3b).

Gomez et al. took advantage of this functionality and inserted the PIF6 protein into the VP2 protein of the AAV nanoparticle capsid (VNP-PIF6).

Figure 3 Light Activated Viral Nanoparticle Using PhyB-PIF6 Heterodimerization. (a) when complexed with PCB, PhyB is considered active (holo-PhyB) and able to absorb red light. Upon absorption, holo-PhyB reversibly binds with PIF6 and this reaction is reversible via dark relaxation of 750 nm illumination. (b) Three separate open reading frames code proteins for the AAV capsid. A VP2 gene modified to include PIF6 at the C-terminus forms a functionalized capsid (VNP-PIF6) when VP1 and VP3 are delivered in trans. (c) Cells are first transfected to express PhyB-NLS and PCB and VNP-PIF6 are added. In the dark or in deactivating light, the virus exhibits minimal basal transduction. Once irradiated with activating red light, PhyB-NLS dimerizes with the exposed PIF6 on the capsid, thus shuttling it to the nucleus and inducing transduction and subsequent transgene expression. Reprinted with permission from Gomez, E. J., Gerhardt, K., Judd, J., Tabor, J. J., & Suh, J. (2015). Light-Activated Nuclear Translocation of Adeno-Associated Virus Nanoparticles Using Phytochrome B for Enhanced, Tunable, and Spatially Programmable Gene Delivery. ACS Nano, acsnano.5b05558. https://doi.org/10.1021/acsnano.5b05558. Copyright (2018) American Chemical Society

Gomez et al. took advantage of this functionality and inserted the PIF6 protein into the VP2 protein of the AAV nanoparticle capsid (VNP-PIF6). Capsid modifications
typically demonstrate diminished basal transduction due to interruption of the native cellular infiltration and nuclear localization machinery on the external surface of the virus. When coupled with expression of PhyB fused to a nuclear localization sequence (NLS) from a plasmid delivered in trans, VNP-PIF6 is shuttled into the nucleus upon irradiation with 630 nm light (Figure 3c). In this way, experimental results in HeLa cells demonstrated a >15-fold dynamic range and a 6-fold greater transduction index (TI) as compared to wild-type AAV. The group further demonstrated that VNP-PIF6 is capable of light-induced transduction in other mammalian cell types such as human MSCs, human embryonic kidney (HEK) 293T cells, human umbilical vein endothelial cells, and mouse 3T3 fibroblasts. Robust activation in the red/far-red regime suggests the VNP-PIF6 may be a good candidate for light-induced growth factor expression for tissue engineering applications. It was therefore selected as a tool for the first aim of this thesis.

7.5.1.5. BphP1-PpsR2

Bacteriophytochromes (BphPs) exhibit the aforementioned ideal criteria better than any other currently characterized light responsive proteins. Canonically, BphPs default in the dark to a FR light (660-700 nm) absorbing state, denoted as P$_r$ in the literature. BphP1, however, is from a family of BphPs known as bathyBphPs which, in the dark-adapted state, absorb near-infrared (NIR) light (740-780 nm), otherwise referred to as P$_{fr}$ (Figure 4a). NIR illumination stimulates BphP1 to enter the P$_r$ state while FR illumination or dark relaxation returns the protein to its relaxed state, P$_{fr}$. Further, BphP1 employs the chromophore biliverdin IX$_{\alpha}$ (BV), a derivative of heme synthesis that is endogenous to mammalian cells, eliminating the need for exogenous chromophore
delivery and allowing a theoretically infinite chromophore supply for long term responsiveness.\textsuperscript{85} The conversion of BphP1 from P\textsubscript{fr} to P\textsubscript{r} has been demonstrated to be dependent upon the applied light intensity and is reversible for at least nine cycles of alternating 740nm/636nm light.\textsuperscript{99} Finally, BphP1 has been shown to heterodimerize with transcriptional regulator PpsR2 presenting the possibility of an optogenetic transcription control system similar to those developed with other light-sensitive proteins (Figure 4c).\textsuperscript{100}

To adapt the BphP1-PpsR2 system to control transcription, Kaberniuk \textit{et al}. took an approach similar to the PhyB-PIF6 system mentioned above (Figure 5b).\textsuperscript{99} BphP1 was fused at the C-terminus to an mCherry fluorescent reporter which was then fused to a TetR DNA binding domain. The composite three-part protein was placed under control
of a constitutive \( P_{\text{CMVd1}} \) promoter and integrated into the genomes of HeLa cells. A second construct was developed that constitutively expressed \( (P_{\text{CMV}}) \) PpsR2 with an N-terminal NLS and a C-terminal transcription activation domain, VP16. The NLS-PpsR2-VP16 plasmid, along with plasmid containing a secreted embryonic alkaline phosphatase (SEAP) reporter protein under control of a Tet-inducible promoter \( (P_{\text{Tet}}) \), were transiently transfected into the modified HeLa cells. In this way, the group achieved a 40-fold activation of SEAP expression in response to activating 740 nm light. Further, the group demonstrated \textit{in vivo} activation of the system up to 5.7-fold in mice livers with ambient 740 nm light, indicating promise for the system in tissue engineering.

This system was further improved by Redchuk \textit{et al.} in 2017 where they identified the minimal portion of the PpsR2 protein required for complexation with BphP1 (Figure 5c).\textsuperscript{101} This functional fraction of PpsR2 is outlined in red in Figure 4a and contains the Q-linker and first Per-Arnt-Sim (PAS) domain, thus is called Q-PAS1. In their optimized approach, Redchuk \textit{et al.} fused the transcription activating domain, VP16, to BphP1 and fused a Gal4 DNA binding domain and an NLS to the Q-PAS1 domain. This improved system almost halved the size of DNA constructs required for system operation yet maintained >40-fold activation and even increased the kinetics of the system, which they hypothesized was because the smaller Q-PAS1 was more quickly transcribed and translated and limited the formation of tetramers and octomers.\textsuperscript{101} Optogenetic systems requiring the least amount of DNA are ideal in that they are more easily cloned, more easily transported across cell and nuclear membranes, and more easily integrated into genomes. The final 3.7 kbp construct is currently the optimal
system to explore what optogenetics can provide for tissue engineering due to its absorption window, endogenous chromophore, and reversible control. This system was selected to implement in the second specific aim of this thesis.

7.6. Research Aims and Studies

7.6.1. Specific Aim 1 – Demonstrate functionality of VNP-PIF6 as a tool for tuned growth factor delivery.


7.6.1.1.1. Objective

In this aim, we sought to optimize protocols for using a modified AAV viral nanoparticle (VNP) for light activated delivery of transgenes, specifically those that...
might be relevant for tissue engineering. We hypothesized that the VNP-PIF6 would allow for robust activation of transgene expression in mammalian HeLa cells once parameters were optimized.

7.6.1.1.2. Experimental Design

Plasmids were created to ensure packaging of desired transgenes by wild-type (WT) AAV serotype 2 (AAV2) and VNP-PIF6. These viruses were quantified and then structurally and functionally validated. Experiments were then employed to measure the light-response of VNP-PIF6. Experimental conditions were run in duplicates in 24-well plates.

7.6.1.1.3. Evaluation

First, the ability to produce a fully formed viral capsid functionalized with PIF6 was assessed. It was then critical to ensure a basal capacity for transduction of the viruses. We then explored how different parameters such as chromophore concentration, light intensity, and light duration impacted the ability to control transduction with applied light conditions. Viruses were evaluated for the magnitude and fold-change of transduction in response to various light conditions. Optimal behavior would include a large dynamic range (~10-fold) with the ability to precisely and consistently tune transduction across that dynamic range.

7.6.1.1.4. Materials and Methods

7.6.1.1.4.1. Plasmids and Assembly

Plasmids required for the assembly of both wild-type AAV and the VNP-PIF6 were previously validated in the laboratory of Dr. Junghae Suh. For Study 1.1 we constructed the plasmids outlined in Table 2. We employed advanced quick
assembly (AQUA) to incorporate our various inserts into the destination vector containing the inverted terminal repeat (ITR) sites necessary for packaging into the AAV viral nanoparticles. Green fluorescent protein (EGFP), generously provided by Dr. Junghae Suh, was used for initial characterizations as it allows high throughput analysis via flow cytometry as compared to other proteinaceous outputs. (BMP2) was generously donated by Dr. Yasuhiko Tabata from Kyoto University. A P2A self-cleaving linker was employed to create constructs with the growth factors cleavably-linked to the EGFP fluorescent protein via annealing driven by ~30bp homologous ends.

7.6.1.4.2. qRT-PCR Assay Development and Implementation

To quantify BMP-2 expression, we designed qRT-PCR primers using the Primer Wizard in Benchling with an optimal GC content of 50%, melting temperature of 60°C, primer length of 22 bp, and amplicon length of 100 bp. We selected the best two output primer pairs for and optimized PCR cycling conditions for melting temperature and primer concentration with a serial dilution created of plasmids pITR-BMP2 ranging from $10^3$ to $10^{13}$ copies/mL. The primer pair that displayed the greatest amplification efficiency and highest fidelity in melt curve peaks was selected for all subsequent qRT-PCR analyses. First, cells were detached from the plate with TrypLE Express (Thermo Fisher Scientific) and the enzyme was neutralized with serum-containing Dulbecco’s Modified Eagle Medium (DMEM). Cells were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 2000 rpm for 5 minutes. Cell pellets were then resuspended in Buffer RLT and RNA was extracted following the RNEasy Kit (Qiagen). RNA concentration and purity were
analyzed with a NanoDrop 1000 spectrophotometer and 10 µL of each sample was added to a reverse transcription reaction to make cDNA using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific). Product of cDNA synthesis was analyzed with the NanoDrop and diluted to 5 ng/µL in sheared salmon sperm DNA (Thermo Fisher Scientific). qPCR reactions were then run with Power SYBR Green (Thermo Fisher Scientific) and the transcript concentration was back-calculated from a regression of the standards and their corresponding threshold cycle.

7.6.1.4.3. Virus Preparation

To produce WT AAV2 packaging different transgenes, HEK 293T cells were co-transfected by polyethylenimine (PEI) with a rep-cap encoding plasmid (pXX2), an adenoviral helper plasmid (pXX6-80), and the respective cargo vector (outlined in Table 2) at a ratio of 1:2:1. To produce the PIF-functionalized viruses (VNP-PIF6), pXX2 was replaced with one plasmid encoding the unmodified VP1 and VP3 (pRC_RR_VP1/3) proteins and one plasmid encoding the PIF-modified VP2 protein (pVP2A-PIF6) at a 4:1 ratio. Cells were harvested and lysed 48 hours after transfection and the cell lysate was applied to a gradient of iodixanol solutions to allow for isolation of viruses via density separation under ultra-centrifugation. Viral titer was quantified via qPCR using Power SYBR Green (Thermo Fisher Scientific) with primers specific to the cytomegalovirus promoter (P_{CMV}) driving expression of the transgene in all packaged plasmids (P_F = TCACGGGGATTTCGAGTCTC, P_R = AATGGGGGGAGTGGTGTTACGAC). Viral capsid proteins were also validated via western blot.
7.6.1.4.4. **Transduction Assay**

To test the basal functionality of the viruses produced we ran a simple transduction assay. HeLa cells were plated at roughly 80,000 cells/well in 24-well plates. Approximately 24 hours later, viruses were added to cells in serum-free media DMEM at three different multiplicities of infection (MOI). Wells were supplemented with DMEM containing serum after four hours. Analysis of output expression was carried out 48 hours after addition of virus either by flow cytometry (for GFP) or qRT-PCR (BMP-2). Fluorescence was quantified in a TI and growth factor expression was quantified as transcripts/mL and compared to unmodified cells to achieve a fold change.

7.6.1.4.5. **Flow Cytometry**

EGFP fluorescence was analyzed with a BD FACScan flow cytometer. Cells were activated with a blue (488 nm, 30 mW) solid-state laser. A 510/21 nm filter (FL1) was employed to observe the emitted fluorescence. For each sample, 10,000-20,000 events were collected and forward scatter (FSC), side scatter (SSC), and green fluorescence (FL1) were noted. To analyze data, .fcs files were run through CytoBank Community and cell populations were manually gated to remove debris events. The output of unmodified cells in the FL1 channel was used to set an autofluorescence threshold where any event with more fluorescence than the threshold is considered a GFP-expressing cell. With this gate, %GFP$^+$ cells per sample was calculated and the geometric mean of these fluorescent cells was extrapolated by the software.
7.6.1.4.6. LED Calibration and Light Program Design

To apply precise light intensities in a high throughput and robust manner, we employed the Light Plate Apparatus (LPA) designed in the Tabor Laboratory at Rice University. In brief, the device applies precisely programmed light intensities and temporal patterns from two LEDs into individual wells of 24-well plates with clear bottoms and opaque well walls. This allows for 24 unique and isolated light conditions to be applied in a single experiment. For the VNP-PIF6 system, 24 each of 630 and 735 nm LEDs were purchased and installed into the LPA (LEDtronics, #L200CWRGB2K-4A-IL). For calibration of the LEDs, all input parameters were set to universal and constant values and the flux from each LED was assessed via a fiber optic photodetector probe and integrating sphere (StellarNet Inc., photodetector #EPP2000 UVN-SR-25 LT-16, probe #F600-UV−vis-SR). The direct current gain was then set to achieve the desired range of output fluxes and the calibration parameter was adjusted for every LED, normalized to the dimmest LED. The flux of each LED was then obtained again and the process repeated until the coefficient of variation between the LEDs was <3%.

7.6.1.4.7. Light Activated Transduction Experiments

Light experiments were performed using black, clear-bottom plates and the LPA discussed previously. Plates were coated with poly(L-lysine) (PLL) to ensure cell-adhesion and HEK 293T or HeLa cells were then plated at 120,000 or 100,000 cells/well, respectively. When cells were about 75% confluent, pKM017 was transfected into the cells with linear PEI at an N:P ratio of 20 in serum-free DMEM, where the N:P ratio is the ratio of amine groups in the linear PEI to the phosphate
groups on the DNA backbone. Four hours later, the transfection mixture was removed and DMEM with serum was added to wells. All steps from this point forward were performed in the dark with an orthogonal green safelight. After 24 hours, media was removed and 300 µL of media, either plain or supplemented with the chromophore PCB at 15 µM, was added to the wells. The plate was then placed in the incubator for one hour to allow PCB to complex with PhyB-NLS. After this hour incubation, virus was added at an multiplicity of infection (MOI) of 2000 in 300 µL media with serum and the plate was placed upon the LPA to undergo light treatment. Light programs lasted 1-12 hours and the plate either remained on the LPA in darkness or was moved off the LPA and covered in foil until 48 hours after addition of virus. After 48 hours, cells were harvested with TrypLE Express (Thermo Fisher Scientific) and analyzed by flow cytometry (GFP) or qRT-PCR (BMP-2).

7.6.1.5. Results

7.6.1.5.1. VNP-PIF6 forms successfully and demonstrates decreased basal transduction as compared to wild-type

Titers from qPCR analysis of virus yielded ~10^{12} WT AAV and ~10^{11} VNP-PIF6 (Figure 6a). This decreased titer as compared to WT is characteristic of viruses comprised of modified capsid proteins. Western blot against the three capsid proteins (VP1, VP2, and VP3) show all three present in the WT AAV (Figure 6b). Further, the increased size of the VP2 protein in VNP-PIF6 suggests the incorporation of PIF6. Basal transduction experiments showed successful transduction by both WT and VNP-PIF6 with expected lower transduction by the modified VNP-PIF6 (Figure
We further validated that our fused BMP2-P2A-EGFP reporter displayed similar results by changing MOI and observing BMP-2 expression via qRT-PCR (Figure 7b). AAV vectors modified in similar capsid locations from other labs have similarly shown decreased basal infectivity, probably due to interruption of cell endocytosis or nuclear localization machinery present on capsid surface.102

Figure 6 VNP-PIF6 forms successfully. (a) Results from qRT-PCR show viral titers (viral genomes/mL) of two representative virus preparations. VNP-PIF6 produced, while fewer than WT, a reasonable amount for experimental use. Error bars are standard deviation, n = 3. (b) Western blot of WT and VNP-PIF6 against capsid proteins show a positive stain for all three. For VNP-PIF6, VP2 is longer, suggesting the insertion of PIF6 as expected.

7.6.1.5.2. PCB fabricated in-house shows predicted behavior

Heterodimerization of PhyB and PIF6 is dependent upon the functional presence of the chromophore PCB. To confirm functionality, we first observed the absorption spectrum of our stock and compared it to spectra from the literature (Figure X). The curve displays the expected bimodality with peaks at roughly 350 nm and 680 nm. To further convince ourselves of PCB function, we implemented a modified yeast line made and validated in house that fluoresces when conjugated with PCB (data
not shown). From these two experiments, we were certain that our stocks of PCB were working correctly.

7.6.1.1.5.3. Transfection and transduction were validated and optimized

Due to the immense parameter space in the composite experimental design, it was imperative to validate each step in the protocol, starting with experimental technique. First, the transfection protocol was optimized for efficiency and cell health. Two transfection methods were investigated, one to improve transfection efficiency and one to improve cell health (Table 1), were investigated at different seeding densities. Method A resulted in greater transfection efficiencies at a constant N:P ratio of 20 as compared to Method B (Figure S2). We predict this is because Method A implemented serum free media as opposed to complete media used in Method B. It was also revealed that lower initial seeding densities (specifically 60,000 cells/well) yielded greater transfection efficiencies regardless of the method.

Figure 7 Validation of Function of WT and VNP-PIF6 Virus Preparations. (a) Total EGFP (as TI) for both virus types at two denoted MOIs suggest successful transduction and transgene expression. (b) Dual reporter transgene is confirmed by qRT-PCR quantification of BMP-2. In both, expected behavior is seen where VNP-PIF6 exhibits lower basal transduction as compared to WT and increasing the MOI yields increased transgene expression. Error bars are standard deviation, n = 2.
(Figure S2). This was optimal for our experimental design anyway, for experiments required sequential transfections and transductions thus extending the total time for cells to propagate on the plate. While Method B did demonstrate improved cell health, Method A yielded sufficient living cells after 24 hours for the subsequent transduction. We therefore chose to proceed with an initial seeding density of 60,000 cells/well and transfection Method A.

We then validated the viral transduction protocol along with our output assays for fused transgene expression. Using a WT and a VNP-PIF6 virus delivering only EGFP, we transduced HeLa cells at various MOIs. For each experimental group, the TI was calculated.

We expect TI to be linear in response to changing the MOI. Ratios of TI suggest very close to a scaled relationship between TI and MOI (Figure S3). Further, TI plotted as a function of MOI yields a tight linear fit ($R^2 = 0.99996$ for WT and $R^2 =$

<table>
<thead>
<tr>
<th>Method A</th>
<th>Method B</th>
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| 1. Make transfection mix  
   • DNA + PEI in serum free media  
   2. Incubate at RT for 20 minutes  
   3. Remove media from wells to be transfected  
   4. Add 300 µL of transfection mix to each well so that 0.125 pmol of DNA is added to each well  
   5. Place plate in incubator for 4 hours  
   6. Remove from incubator and aspirate transfection mix  
   7. Add 600 µL complete DMEM and place in incubator and analyze via flow cytometry 48 hours later | 1. Change media on wells to be transfected  
   2. Four hours later, make transfection mix  
   • DNA + PEI + NaCl in sterile H$_2$O  
   3. Vortex and incubate at RT for 30 minutes  
   4. Add 23 µL directly into each well  
   5. Place in incubator and analyze via flow cytometry 48 hours later |
0.95984 for VNP-PIF6). From these data, we can conclude that the technique is sound and that our viruses and transgenes exhibit expected basal functionality.

7.6.1.1.5.4. **Light induction experiments yield minimal success in our hands**

Once our protocols were optimized for transfection and transduction efficiency, along with cell health, we ran the full light induction protocols. Our initial experiments in HEK 293T cells were erratic when comparing raw TI values, but appeared to follow the predicted trend when normalized to the WT control used in every light experiment. This accounted for any timing or cell health variability that occurred between experimental runs (Figure 8).

![Figure 8](image)

**Figure 8: EGFP Expression in HEK 293T Cells Tuned by Applied Light Condition.** HEK 293T cells transduced with pITR-EGFP. When experimental conditions are normalized to WT EGFP a direct relationship between applied R:FR ratio and output TI is visible. Normalization is calculated by dividing TI achieved by VNP-PIF6 under specified light condition by TI achieved by WT in unmodified cells in the dark. This accounts for variation in experiment timing between days. FR flux set to 0.00125 µmol/m²s and R light tuned accordingly to achieve denoted R:FR ratio.

While promising, our maximum induction values were significantly less than WT, even when delivering the EGFP-only transgene. Previous results suggested that
VNP-PIF6 could demonstrate 6X induction of transgene delivery as compared to WT. Despite this shortcoming, we then explored the function of VNP-PIF6 in HeLa cells to demonstrate robust function. Unfortunately, we were unable to achieve the same light induction profile that we saw in HEK 293T cells. New viruses were prepared and validated in the same manner as previously described. Perplexingly, experimental results suggested that the addition of PCB significantly increased the TI of both the WT and VNP-PIF6 viruses while the addition of the dimethylsulfoxide solvent did not (Figure S4). It also appears that transfection is dependent upon the concentration of PCB (Figure S4). No experiment in HeLa cells resulted in any measurable light-dependence of VNP-PIF6 even in multiple hands and we saw evidence that further increasing the applied 630 nm light flux does not confer light activation to the VNP-PIF6 tool. We finally demonstrated that the duration of applied light does not impact the function of VNP-PIF6 in a positive way (Figure S5).

7.6.1.1.6. Discussion

VNP-PIF6 represents an innovative and advantageous tool for the optically induced transduction of therapeutic transgenes. The ability to tune output expression in magnitude and space represents a significant advantage over scaffold based and even previous gene therapy methods. Further, the potential for the VNP to achieve greater than WT transduction suggested that it could easily achieve physiologically relevant levels of transgene expression at lower viral titers, thus improving the safety profile of the virus. We were, however, unable to achieve these characteristics in our approach. While we did validate many components of the protocol and system, there
were a few stones left unturned. First, we did not confirm the presence of functional PIF6 exposed on the capsid of VNP-PIF6. While the western blot indicated an increased size of VP2, there was no functional analysis confirming that PIF6 was accessible on the exterior of the capsid or that it was functional for dimerization with PhyB. It has been documented that the virus purification protocol can deplete or eliminate the function of any proteins incorporated into the capsid, a possible cause of the poor functioning we saw. Another potential source of error that was not addressed here was the presence or absence of functional PhyB-NLS. qRT-PCR indicated the presence of PhyB mRNA (data not shown), however we did not confirm the presence of functional protein produced by the transfected cells. Since we used a plasmid validated not only by Gomez et al. in 2016 but also by Muller et al in 2013, it is unlikely that the PhyB-NLS produced is non-functional. It is a possibility, however, that our transfection method did not yield enough of the protein to result in significant nuclear translocation of VNP-PIF6. The molar ratio of PhyB-NLS proteins to each VNP-PIF6 is unknown and we might have been achieving expression below that threshold.

Previous results suggest that VNP-PIF6 could be a tool for optimized growth factor expression for tissue engineering. It is responsive to relatively long wavelengths of visible light (630 nm) which can penetrate tissue well compared to other visible wavelengths. Further, the response is tunable by the intensity of applied light allowing for control over delivered dose. It can also be controlled spatially via light masks. Even had we seen success in our hands, there are a few key weaknesses to the VNP-PIF6 system that diminish its potential for tissue engineering. First, the
light-induced dimerization of PhyB with PIF6 requires the addition of an exogenous chromophore. This limits the functionality of the system to working only while PCB is present. In actual clinical settings, PCB localization and degradation would be barriers to successful utilization. Second, this tool achieves light-activated transduction of a constitutively expressed transgene, thus offering no temporal control of expression apart from initiation. Similar tunability could be achieved through changing the MOI, using a chemically inducible system, or simply delivering a known concentration of growth factor over an extended time, thus offering minimal advantage over current methods apart from spatial resolution. For these reasons, we transitioned to exploring a light-responsive transcription control system in Specific Aim 2.

7.6.2. **Specific Aim 2 – Demonstrate functionality of BphP1-PpsR2-based transcription control systems for tuned growth factor delivery**

The goal of the second specific aim was to test and optimize experimental protocols for light-controlled transcription of a GOI using the BphP-PpsR2 heterodimerizing pair. From this aim, we desired a tool for the precise control of growth factor expression over physiologically relevant concentrations. To achieve this, we performed the following studies: (1) Optimize transfection protocols to maximize dynamic range of a BphP1-PpsR2 transcription control system with a fluorescent reporter, and (2) implement the BphP1-PpsR2 systems to control VEGF expression

7.6.2.1. **Study 2.1 – Optimize transfection protocols to maximize dynamic range and repeatability of a BphP1-PpsR2 transcription control system**
7.6.2.1.1. **Objective**

The objective to Study 2.1 was to validate and optimize experimental protocols for the BphP1-PpsR2 transcription control system generously provided by the Verkhusha Laboratory at Albert Einstein College of Medicine. For this study, all experiments and data were performed with the first generation of the transcription control system (Figure 5b). Briefly, we employed a HeLa cell line that stably expresses BphP1-mCherry-TetR from the genome. We then transiently co-transfected cells with plasmids pCMV-104 (constitutively expressed NLS-PpsR2-VP16) and pTRE-Tight-EGFP (TetR-inducible EGFP reporter plasmid). With this approach, we sought to optimize the consistency and dynamic range of this system by varying the plasmid ratios, transfection parameters, and applied light intensity.

7.6.2.1.2. **Experimental Design**

Each condition was tested in duplicate wells on the opaque-side/clear-bottom plates compatible with light application via the LPA. Experimental conditions undergo 48 hours of their respective optical conditions and output was assayed via flow cytometry.

7.6.2.1.3. **Evaluation**

The BphP1-PpsR2 system was assayed for its ability to induce transgene expression under the various conditions tested. The performance of the system was assayed via flow cytometry on cells expressing EGFP under control of the optogenetic system. Data was analyzed for consistent, significant differences between various applied light intensities with the end goal being to maximize the dynamic range.

7.6.2.1.4. **Materials and Methods**
7.6.2.1.4.1. Plasmid and Cell Line Verification

Modified HeLa cells were gifted from the laboratory of Dr. Vladislav Verkhusha. Stable HeLa cells expressing BphP1-mCherry-TetR, hereafter called HeLa-BmCTs) were validated via fluorescence microscopy for mCherry expression. Cells were imaged once a week to validate long term expression of the integrated expression unit after many successive passages. Similarly, plasmids were also donated by the Verkhusha Laboratory. For this aim, we worked primarily with pCMV-104 and pTRE-Tight-EGFP. Plasmid sequences were verified via Sanger Sequencing at Genewiz ® in South Plainfield, New Jersey.

7.6.2.1.4.2. Co-transfection and Light Activation with Various Methods

Initial transfections were performed using a linear PEI as with Specific Aim 1. In brief, each well of an LPA plate was coated with 0.001% PLL suspended in phospho-buffered saline (PBS) to encourage cellular attachment. Then HeLa-BmCTs were plated at roughly 100,000 cells/well and left in the incubator. When wells reached ~75% confluency, media was removed and the transfection mixture was added. To make the transfection mixture, pCMV-104 and pTRE-Tight-EGFP at a w/w ratio of 5:1 were combined with PEI in serum free media and allowed to incubate at room temperature for 20 minutes. The N:P ratio was optimized in the results section. 300 µL of the DNA+PEI solution was then added to wells such that the entire surface was in contact with media. The total mass of DNA was 0.75 µg/well unless otherwise noted. After four hours of dark incubation, 300 µL of media containing serum was added to each well and the plate was placed on the LPA. For the BphP1-PpsR2 transcription control system, optimal light patterns were
empirically found to follow a 30s ON/180s OFF cycle. Light conditions were applied for 48 hours at which point cells were harvested for flow cytometry.

7.6.2.1.4.3. Flow Cytometry

Flow was run similarly to in Specific Aim 1 with new gates made for the HeLa-BmCT size and autofluorescence.

7.6.2.1.5. Results

7.6.2.1.5.1. Full-length BphP1-PpsR2 exhibits light activation and tunability in HeLa-BmCT cells.

We first investigated the full length BphP1-PpsR2 system using the HeLa-BmCT cell line that stably expresses BphP1-mCherry-TetR from the genome. The PEI transfection protocol (Method A in Table 1) yielded >80% transfected cells when using a constitutive GFP reporter (Figure S6). At first pass, we saw minor activation. We then explored the effect of the ratio of co-transfected plasmids. A 2:1 ratio resulted in the largest magnitude of expression while a 5:1 ratio resulted in the

![Figure 9](image.png)

**Figure 9 Effect of Co-transfection and N:P Ratios on System Response.** Black bars were in dark, light brown/red bars are FR = 25 µmol/m²s, dark brown/red bars are 50 µmol/m²s, green marks are the event rate as measured on the flow cytometer. Error bars are standard deviation, n = 2. (a) Ratio of pCMV-104:pTRE-Tight-EGFP (w:w) has a significant impact on system response. The recommended 5:1 ratio resulted in the greatest fold change and lowest background. (b) N:P ratio of PEI transfection impacted system performance and cell health.
lowest background EGFP expression and greatest fold-change (Figure 9a). We therefore continued forward with the 5:1 ratio since an optimal system for therapeutic factor delivery would have a robust off state to avoid negative side effects associated with overexpression. In the same experiment, we observed that increasing the applied light flux increased the resulting EGFP output. We then explored the effects of altering different transfection parameters on the resulting EGFP expression while further increasing activing light flux. The first parameter space explored was the N:P ratio with the goal being to increase the output dynamic range and improve cell health (Figure 9b). As expected, increasing the N:P ratio resulted in lower cell health due to membrane disruption. As a metric for cell health, we used the event rate of samples analyzed on a flow cytometer. Since every sample was resuspended in the same amount of media for analysis, the event rate corresponds to the concentration of cells in the solution. Further, since all wells were plated at the same density, the concentration of cells at analysis is representative of relative cell health. Fortunately, we saw the greatest magnitude and fold-change of induction at the lowest N:P ratio tested (Figure 9B). We therefore conducted all following experiments at an N:P ratio of 17. Even at this optimized ratio, however, we saw cell viability issues.
We next compared the two transfection methods in Table 1 to determine if improved cell health would improve the function of the system. Concurrently, we increased the applied light flux with the goal being to achieve maximum induction output. Results suggested that, while Method B does improve overall cell health, the magnitude of induced EGFP expression is significantly decreased when compared to Method A (Figure 10a). Interestingly, the fold change remained similar between methods. We therefore continued forward with Method A to achieve maximal transgene expression.

Further increasing the applied 730 nm flux resulted in an even greater EGFP output while following almost identical curves between experimental runs, suggesting that the system is precisely repeatable and that we were still not employing the full dynamic range (Figure 10b). We finally explored the impact of improved transfection efficiency on system performance by using FuGene HD Transfection Reagent. With FuGene, we achieved a dynamic range of over 5-fold upon 730 nm...
activation and a 2-fold increase over the maximum output under Method A transfections (Figure 11). In all, we determined that the BphP1-PpsR2 system was functional and repeatable and that transfection parameters play a significant role in the function of the system.

7.6.2.2. Study 2.2 – Implement the BphP1-PpsR2 system to control growth factor expression

7.6.2.2.1. Objective

The objective of Study 2.2 is to take the experimental protocols developed in Study 2.1 and use them to achieve and optimize expression of therapeutically relevant growth factors. Experiments in this study control the expression of VEGF with an end goal being to optimize VEGF expression profiles for improved vasculature of engineered tissues. In this study, we not only explored the utility of the BphP1-PpsR2 system validated in Study 2.1, but we also explored the use of the second-generation

![Bar chart showing Geometric Mean of EGFP+ Cells under different N:P and F ratios](image)

Figure 11 Increasing Transfection Efficiency via FuGene HD Improves System Response. The use of a commercial transfection reagent resulted in significantly increased transfection efficiency as compared to our PEI Method A transfection (N:P = 17). This subsequently increased the magnitude and dynamic range of the BphP1-PpsR2 system in response to 180 µmol/m²s 730 nm light.
transcription control system that employs the smallest functional fraction of the PpsR2 protein (Figure 5c). In all, we sought to compare the two systems in their ability to produce a wide range of VEGF concentrations that span a therapeutically relevant region.

7.6.2.2.2. Experimental Design

Experimental protocols and light conditions optimized in Study 2.1 were used to induce expression of VEGF from the inducible promoters. Groups were assayed for the final concentration of secreted VEGF in the media after 48 hours via enzyme linked immunosorbent assay (ELISA). Each experimental condition was run in duplicate and each sample was analyzed via ELISA in duplicate, yielding four concentration values for each experimental condition.

7.6.2.2.3. Evaluation

To be useful for tissue engineering, this system must induce protein expression at physiologically relevant levels. This depends highly upon the method of VEGF administration or delivery, but literature indicates this functional concentration to be on the order of 0-500 pg/mL/day. The system therefore was evaluated by the magnitude of the VEGF output and the dynamic range of the output.

7.6.2.2.4. Materials and Methods

7.6.2.2.4.1. Cloning of VEGF reporter plasmids

Reporter plasmids were built using Golden Gate cloning. Backbones containing TetR and Gal4 inducible promoters were extracted from pTRE-Tight-EGFP and pU5-Rluc8 respectively via PCR amplification. Vascular endothelial growth factor (VEGF) was purchased as rhVEGF121 from the laboratory of Dr. Michael Grusch.
rhVEGF<sub>165</sub> was built by creating 60bp oligomers with 15bp overlaps to create a fragment containing the additional DNA. Final VEGF<sub>165</sub> was then placed in two plasmids under control of the two inducible promoters. pTP015.3 and pTP017.4 were thus TetR inducible and Gal4 inducible rhVEGF<sub>165</sub>, respectively. A third reporter plasmid was made with TetR inducible VEGF-P2A-EGFP (pTP015.2). The two fused proteins were combined via AQUA ligation. For experiments using the modified BphP1-Q-PAS1 system (Figure 5c) plasmid pQP-T2A was used to deliver BphP1-VP16 and NLS-Gal4-QPAS1 on the same plasmid co-expressed from P<sub>CMV</sub>.

7.6.2.2.4.2. Co-transfection and Light Activation

For the system implementing the full length PpsR2, HeLa-BmCT cells were transfected as in Study 2.1. For the BphP1-Q-PAS1 system, however, unmodified HeLa cells were used instead of the HeLa-BmCT cells, as the full system is included in the plasmid. For co-transfection with pQP-T2A and pTP017.4, the plasmids were delivered at a 5:1 ratio (w/w) and a total of 0.75 µg/well. After 48 hours of applied light conditions, media was collected from each well and placed in 1.5 mL microcentrifuge tubes for storage at -80°C. Cells were also collected, spun down, and stored as pellets in the -80°C freezer for qRT-PCR analysis. Samples were analyzed via ELISA against VEGF per manufacturer’s instructions (R&D Systems, DY293B). A four-parameter logistical fit was created with the supplied standards and used to calculate the concentration of VEGF in the media following the 48-hour light program. Each experimental condition was run in duplicate for light experiments and each sample was then run in duplicate for ELISA, yielding four
concentrations per experimental group. Data points are thus an average of the four total ELISA wells per experimental condition and statistics were performed on the four points.

7.6.2.2.5. Results

7.6.2.2.5.1. Full-length BphP-PpsR2 and minimal BphP1-QPAS1 achieve optically tuned VEGF expression.

With a working optogenetic system at our disposal, we moved forward to control of therapeutic gene expression with an VEGF-P2A-EGFP reporter using the Method A transfection protocol in HeLa-BmCTs at an N:P ratio of 17. Fluorescent images indicated successful EGFP expression and flow cytometry results suggested light induction, however EGFP signal was minimal. Interestingly, the fused reporter yielded a similar fold change between highest and no activation conditions but at a significantly decreased magnitude of expression (Figure 12). Cells were analyzed for mRNA levels of VEGF using validated primers. qRT-PCR results demonstrated

![Figure 12 Fused Reporter Shows Tuned Fluorescence but not Tuned VEGF.](image)

(a) Flow cytometry analysis of HeLa-BmCT cells cotransfected with pCMV-104 and pTRE-Tight-VEGF-P2A-EGFP shows tuned fluorescence in response to 730 nm light (units µmol/m²s). The magnitude of this response is much less than with the only EGFP reporter. (b) qRT-PCR analysis against VEGF mRNA (Supplemental Information) shows promising but inconclusive results due to the aberrant data at 87.9 µmol/m²s. Error bars are standard deviation
close to predicted patterns but with minimal dynamic range and significant deviation (Figure 12). We therefore moved away from the dual reporter system and explored quantifying a single VEGF output. Further, the most convincing argument for a useful optogenetic system would be a functional output, necessitating a switch to quantifying secreted VEGF protein as opposed to mRNA levels.

Figure 13 Both Bph1-PpsR2 and BphP1-Q-PAS1 Induce Physiologically Relevant VEGF Secretion. (a) Full length BphP1-PpsR2 reaches physiologically relevant VEGF concentrations and sees significant activation at 180 µmol/m²s of activating FR light flux. Dotted red line indicates Basal VEGF levels of HeLa-BmCT cells. (b) Minimal BphP1-QPAS1 achieves similar activation and expression in HeLa cells. Dotted red line indicates unmodified HeLa cells. In both, error bars are standard deviation. Groups that do not share a letter are significantly different, p<0.01 by ANOVA post hoc Tukey’s HSD.
For functional VEGF output, we analyzed the secreted protein concentrations in media via ELISA. Light experiments were run via Method A at an N:P of 17 and the same light programs as with the EGFP reporter experiments. With the BphP1-PpsR2 system in HeLa-BmCTs, ELISA results for maximum 730 nm light (188 µmol/m²s) indicated a 2.30-fold increase in secreted VEGF protein as compared to dark conditions. These concentrations ranged from a basal output of 150 pg/mL/48hrs to 350 pg/mL/48hrs. With the BphP1-QPAS1 system in HeLa cells, we saw close to a 5-fold increase in maximum 730 nm light as compared to dark conditions with concentrations ranging from 50 pg/mL/48hrs to 250 pg/mL/48hrs. Controls showed only a slight increase in reporter only conditions indicative of promoter leakiness.

7.6.2.2.6. Discussion

BphP1-PpsR2 represents a near-ideal system for optical control of transcription for tissue engineering. Via a chromophore endogenous to mammalian cells, the two proteins dimerize in response to FR/NIR wavelengths of visible light, optimizing it for the penetration window of human tissues. Further, the reversible control would theoretically allow for long-term control over growth factor expression. The identification of the smallest functional unit of PpsR2 (Q-PAS1) further enhances the system by simplifying its implementation as genetic material and allowing it to respond more quickly due to more rapid production and easier mobility through the cells. Our work sought to take advantage of these strengths to validate this system as a tool for controlled growth factor delivery in bone tissue engineering. Most significantly from our work, we can conclude that both systems (BphP1-PpsR2 and BphP1-QPAS1) are able to tunably induce transcription and subsequent
translation of transgenes. Further, data indicates that this induced VEGF secretion is within physiologically relevant concentrations. This is the first time this protein pair has been used for this purpose. From the ELISA results, we see that the basal output of immortal cells is of a concern in these in vitro assays. Because they are immortalized cancer cells, HeLas and HeLa-BmCTs should express relatively high basal levels of VEGF, as is the expected phenotype of cancerous cells. It is unclear why basal VEGF levels are different between the two cell lines, however. Further investigations into actual basal level are warranted. It would also be prudent to explore cell lines with little to no basal VEGF expression to get a better idea of the actual function of the light system.

7.6.3. Specific Aim 3 – Investigate the feasibility of optogenetic tools in common materials for cell-laden scaffolds

While optogenetics does provide many advantages to the control of growth factor delivery, most cell therapy methods exist in 3D space. Therefore, it is important to explore how materials commonly used in tissue engineered 3D scaffolds interact with applied light. To investigate this, we employed the following study: (1) Characterize how different scaffold fabrication parameters impact the absorbance of light.

7.6.3.1. Study 3.1 - Characterize how different scaffold fabrication parameters impact the absorbance of light.

7.6.3.1.1. Objective

The objective of this study was to explore how scaffold fabrication parameters impact the ability of light to propagate through the bulk of the material. Tissue engineering strategies that leverage cell delivery rely upon scaffolds to define and maintain the
defect space as well as provide structural support to delivered cells as they begin restoring the ECM. Therefore, it is relevant to explore how scaffold traits commonly modified to alter composite scaffold mechanical properties, porosity, swelling, and more impact the ability of light to reach cells within the 3D space.

7.6.3.1.2. Experimental Design

To achieve the described objective, preliminary experiments in this aim employed gelatin as our model for a common transparent and cytocompatible polymer for tissue engineering scaffolds. We explored how crosslinking type, crosslinker concentration, and scaffold thickness impact the absorbance of different light wavelengths.

7.6.3.1.3. Evaluation

For this study, experimental samples were evaluated for their absorptive properties of a spectra of wavelengths by either a spectrophotometer or plate reader.

7.6.3.1.4. Materials and Methods

7.6.3.1.4.1. Scaffold fabrication and absorbance measurement

Various amounts of gelatin (Nitta Gelatin, Inc., G-2554P, Japan) was dissolved in DI water under constant stirring at 60°C. Then, either 1mL of the solution was pipetted directly into cuvettes to allow for physical crosslinking, or the solution was further mixed with various concentrations of glutaraldehyde (GTA) solution, Grade I, 25% (Sigma-Aldrich, G5882-10mL) to induce chemical crosslinks, then pipetted into cuvettes. Once solidified, sample absorbance was analyzed via a spectrophotometer with a wavelength range of 400-700 nm to encompass the activating wavelengths of all current optogenetic systems. To analyze the effect of scaffold thickness, various
volumes of gelatin solution were pipetted into wells of a 24-well plate. Absorbance was then measured on a plate reader with the same 300-800nm wavelength range.

7.6.3.1.5. Results

7.6.3.1.5.1. Key scaffold fabrication parameters have significant impact on absorbance properties of material

With gelatin as a model, we investigated the effect polymer weight percent has on the absorbance properties of scaffolds. First, the innate absorptive properties of gelatin can be observed, as the absorbance is inversely proportional in the range of wavelengths investigated. Next, we can see evidence of an increase in absorbance across all wavelengths tested as the weight percent of gelatin is increased (Figure 14a).

![Graphs showing absorbance properties](image)

**Figure 14 Effects of Crosslinking Method and Concentration on Material Absorbance Properties.** (a) Increasing wt% of gelatin increases absorbance across all wavelengths. (b) Addition of GTA chemical crosslinker yields increased absorbance and a change in spectrum shape. (c and d) Further increasing wt% gelatin while increasing chemical crosslinker further increases absorbance and induces more changes to spectra shape.
We next explored how crosslinking type and density impact the absorbance of the material by inducing chemical crosslinking with GTA. This was the chosen crosslinker due to its ability to form spontaneous covalent bonds with amine groups common to ECM proteins. The final gelatin scaffold takes on a vastly different absorption profile when covalently crosslinked as compared to only physical crosslinks. The absorbance magnitude is significantly increased as a result of the presence of GTA-induced crosslinks. In a 2% gelatin solution, the addition of GTA results in the formation of an absorbance peak at around 500 nm. The influence of GTA on the absorbance spectra becomes more complex as the weight percent of gelatin is increased to 55 and 10% (Figure 14b,c,d). In those cases, three peaks appear between 500 nm and 650 nm while significantly increasing overall absorbance of the material.

Finally, we explored how material thickness impacts the ability of light to travel through the material. Using a single batch of 5% gelatin, various volumes were added to wells of a 24-well plate to achieve a gradient of thicknesses. Figure SX displays the light transmitted as a function of the estimated thickness of gelatin within the wells. Apart from a single discrepancy, the transmitted light has an inverse relationship with the gelatin thickness.

7.6.3.1.6. Discussion

Tissue engineering interventions are dependent upon characteristics of the scaffold used. Mechanical properties, porosity, swelling, and functionalization have all demonstrated significant impacts on the behavior of native or delivered cells and the subsequent success of the material in regenerating functional tissues. These
parameters are tuned to achieve varieties of subsequent cellular phenotypes and, to date, it has not been investigated how changes in these parameters impact the ability of visible light to travel through the scaffold for optogenetic purposes. For optogenetics to be useful to tissue engineering, it is important to characterize the behavior of a chosen system in 3D. Therefore, we performed proof of concept experiments to defend this point and begin the process of characterizing material-light interactions.

A common and simple scaffold parameter altered to tune scaffold mechanical properties is the monomer/polymer weight percent. With gelatin, the presence of greater amounts of protein per volume solvent results in greater interactions, thus a stronger resulting scaffold. We inferred that, along with increasing the physical crosslinking, this would also increase the incidence of optical obstruction. This is confirmed by the data in Figure 14, as adding more gelatin to the same volume solvent increases the absorbance of the composite material. Another method for increasing scaffold mechanical strength is to add a chemical crosslinker, as covalent bonds are much stronger than physical interactions. For our work, we employed GTA at various percentages (0%, 0.1%, and 0.5%). We did explore concentrations greater than this, but crosslinking happened so quickly that GTA did not even fully mix with the gelatin, yielding heterogeneous properties across the material. We hypothesized that the addition of chemical crosslinks would increase the absorption of the material. This is exactly what happened, but what did not predict was the significant impact on the shape of the absorbance spectra produced in the different groups. The appearance of peaks at different wavelengths is of immense
importance to applying optogenetic systems to 3D scenarios. For example, we see an absorbance peak appear at roughly 650 nm in the 5% and 10% gelatin groups upon the addition of any GTA, a wavelength close to the absorption peak of the VNP-PIF6 system. This would limit the ability of light to propagate through the scaffold and thus would impact the ability to optically induce transduction.

Intuitively, we also demonstrated that the thickness of the material impacts the penetration of light. This is of particular importance for tissue engineering, as penetration through the implanted scaffold will be exacerbated by the need to penetrate through tissue. Overall, results from Specific Aim 3 demonstrate that properties of the scaffold and optogenetic system must be considered together when chosen. For any application, the scaffold properties are critical to the formation of new tissue, but must also be optimized for the required activation wavelength and intensity of the optogenetic system to be used. With relatively transparent polymers, optimizing this interplay is realistic; however, if a particular intervention required the use of a more opaque polymer to achieve desired mechanical or biological properties, we envision the selection of optogenetic system to be nontrivial. Further, if an intervention seeks to control the expression of multiple growth factors, the two optogenetic systems chosen must be compatible with the scaffold, thus adding to the complexity of design.

8. **Conclusions**

Growth factor delivery for tissue engineering purposes shows immense promise, but the delivery of potent morphogens yields immature or dysfunctional tissue formation, along with many negative side effects. Recently developed optogenetic systems may hold the key for
precise spatial and temporal control over growth factors. Light and proteins that respond to it display many tunable parameters including wavelength specificity, proportionality to applied intensity, spatial resolution, and temporal dynamics. We first explored the utility of a light-controlled AAV called VNP-PIF6 that takes advantage of one such protein pair, PhyB-PIF6. While we saw some tunable response due to light, the limitations inherent to controlling transduction instead of transcription and the difficulty of use imply a future for the technology outside of tissue engineering. We then tested a transcription control system based upon a different light-inducible pair, BphP1-PpsR2. This system displayed robust activation and tunability in response to light. Further, we demonstrated that it could activate VEGF expression to therapeutic levels, thus defending its place as a useful tool for controlling growth factor expression. Finally, we showed that even the simplest of parameters available to tune scaffold properties impact that scaffold’s ability to propagate or absorb light. Further, these absorbance properties are wavelength dependent, thus necessitating the calculated selection of specific optogenetic systems for specific tasks. Future work needs to explore long term control over VEGF expression, as that is key to successful neovascularization in fracture healing. Further, one can explore a variety of VEGF expression profiles over extended time periods to optimize vessel growth and maturity. As more optogenetic tools are discovered and developed, this toolkit will grow to potentially allow orthogonal optogenetic control over multiple growth factors.
Table 2 – Plasmid names and content

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<th>Promoter</th>
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<td>CMV</td>
<td>BamHI-CMV-Ehancer-CMV-Promoter-HindIII-β-globin-intron-Agel-VEGF_{121}-EGFP-Xhol-β-globin-poly(A)-NcoI</td>
</tr>
<tr>
<td>pKM017</td>
<td>8, S4, S5</td>
<td>pSAM200</td>
<td>SV40</td>
<td>PSV40–PhyB(1–908)–VP16–NLS–pA</td>
</tr>
</tbody>
</table>
9. References


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