Special Issue: Positioning of Tissue Engineering in Regenerative Medicine

Mini Review

Leveraging synthetic biology for tissue engineering applications

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  Restoration of damaged tissues and organs requires precise control of cellular processes at the molecular level. Synthetic biology offers genetic tools that can be used to program the molecular biology of the cell, thereby potentially overcoming the various challenges hampering contemporary tissue engineering applications.

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Introduction

Since its inception, tissue engineering has taken an interdisciplinary approach to design functional replacements for human tissues and organs[1]. The current paradigm for tissue repair and regeneration employs the following three components: cells, bioactive molecules and biomaterials[2]. Because tissue formation demands a high level of control, extensive efforts have been invested in recapitulating the cellular microenvironment and in using an array of physical, chemical, and mechanical cues to promote lineage-specific cell phenotype[3]. Different classes of materials (ex. polymers, metals, ceramics, composites)[2], scaffold configurations (ex. hydrogels, fibrous meshes, microspheres)[2], and substrate topographies (ex. grooves, pillars, pits, nanotubes)[4] have been examined for their ability to promote desired cell behaviors. Likewise, the effects of released growth factors and other therapeutic agents have also received substantial attention. Various types of bioreactors have also been constructed to mimic dynamic conditions encountered by cells *in vivo*. Constructs reminiscent of native tissues seemingly result from these microenvironmental manipulations[5-7], but achieving the requisite compositional and mechanical standards for clinical application nevertheless remains a daunting, largely unpredictable task.

The cell is a dynamic entity that probes its surroundings and responds to the signals to which it is exposed. While tremendous advancements in tissue engineering have resulted from controlling parameters external to the cell, it is becoming increasingly evident that internal cues should be given commensurate consideration. Synthetic biology is a nascent but rapidly expanding field that aims to engi-

neer cellular decision-making processes with genetically-encoded parts that are either sourced from nature or developed in the laboratory[8]. Though most early work was performed in prokaryotes, the recent trend has seen rapid development of tools for mammalian cells, which bears significant implications for eventual clinical translation in tissue engineering applications. This Mini-Review highlights the importance of tissue regulation at the genetic level and how the approaches of synthetic biology can help overcome limitations in contemporary tissue engineering.

Improving control in tissue engineering strategies

Bioactive molecules such as differentiation factors or extracellular hormones are frequently applied externally to coax stem cells down a specific lineage[9-11] or delivered directly to an injury or defect site to stimulate intrinsic repair[12]. Despite their therapeutic effects[13-15], such factors suffer from a number of challenges. Growth factors often require laborious purification protocols and can be quite expensive. Finite amounts are also administered to cells at specific timepoints during treatment, and as a result, the lack of a continuous source of growth factors frequently results in a requirement for supraphysiological concentrations, which does not bode well for clinical application. For half-life extension and more controlled release profiles *in vivo*, many bioactive molecules have been physically adsorbed onto, chemically cross-linked to, or encapsulated within biomaterials[16]. Multiple growth factor delivery, however, still remains challenging because the delivery vehicle imposes limitations on cargo capacity, while the molecules themselves may require different release patterns that can be difficult to accommodate in a single carrier. Biomaterial modifications also introduce a wealth of complications, as there are many parameters that must be taken into account. For example, the degradation kinetics (and hence, resulting growth factor release profile) of a hydrogel depend on polymer type, polymer molecular weight, crosslinking agent, crosslinking density, and environmental factors[17, 18]. Long-term, sustained concentration levels that do not eventually taper off therefore cannot be attained solely through biomaterial-based growth factor release strategies.

Consequently, synthetic biology provides an attractive alternative to growth factor delivery by relying on the cells' own machinery to secrete therapeutically relevant molecules, which commonly entails transfecting or transducing genes of interest to yield genetically modified cells[19-21]. Engineering cellular production mitigates the issues of growth factor supply and molecular cargo capacity. However, the gene of interest must be precisely controlled to avoid unintended consequences and off-target effects. For example, constitutive high-level expression of vascular endothelial growth factor (VEGF) from myoblasts implanted locally in a murine ischemic model has been shown to cause vascular tumors and animal death[22]. Furthermore, unregulated bone morphogenetic protein-2 (BMP-2) expression leads to bone overgrowth and disorganization[23]. The transcription factor Sox9 has also been shown to hamper type II collagen synthesis by primary chondrocytes if over-expressed[24]. Improved regulation of gene expression would thus allow for identification of therapeutic windows that permit highly effective treatments while minimizing complications, which is essential when considering clinical applications.

More precise temporal control will also enhance the architecture of tissue-engineered constructs, by accounting for dynamic gene expression patterns observed in biological phenomena such as cellular differentiation. For example, chondrogenesis—the process in which mesenchymal stem cells (MSCs) transition to chondrocytes and eventually form cartilage and bone—requires Sox9, Sox5, and Sox6 expression during the early chondroprogenitor phase, Runx2, Osterix, and Lef1 at the later hypertrophic stage prior to ossification, and various other transcription factors at intermediate stages[25]. The ability to express and repress specific genes over different timeframes would not only better mimic natural pathways, but could also illuminate underlying mechanistic principles of these cellular processes, which can feed back to serve as a blueprint for subsequent tissue engineering approaches.

As a starting point, synthetic gene regulatory devices provide a powerful interface in which external components can be used to reversibly tune cellular expression levels. Many of these transcriptional control modules are amenable to mammalian cells[26], and rely on chemical inducers, such as antibiotics[27, 28], vitamins[29], food additives[30], or amino acids[31]. One study involved a stable murine MSC line expressing recombinant human bone morphogenetic protein-2 (rhBMP-2) under a doxycycline-repressible system[32]. Following transplantation of these genetically modified cells into critical-size segmental bone defects, mice received doxycycline in their drinking water to inhibit

rhBMP-2 expression[32]. Withholding of doxycycline treatment enhanced bone healing *in vivo* compared to one high-dosage administration of rhBMP-2 growth factor, indicating that cellular regulation was a more effective strategy[32]. However, several instances of bone overgrowth were observed in the absence of doxycycline and highlighted the need for additional fine-tuning of antibiotic concentrations to prevent this side effect[32]. More recently, Yao et al. showed that transfected primary chondrocytes with doxycycline-inducible Sox 9 expression seeded onto poly(3-hydroxy-butyrate-co-3-hydroxyhexanoate) (PHBHHx) scaffolds could effectively maintain cartilage-related extracellular matrix proteins type II collagen and aggrecan, which serves as a good starting point for avoiding chondrocyte dedifferentiation ─ an issue often encountered during *in vitro* cell culture[33]. These outcomes reiterate the advantage of gene therapy over growth factor treatment, and that regulation at the molecular level is essential. Extending this concept from single-gene to multi-gene control, one could envision adding various combinations of inducers to control the levels of transcription factors involved in different stages of stem cell differentiation.

However, a potential drawback of using small molecule ligands is that they are subject to complex pharmacokinetics in the human body, and that switching between a gene's ON/OFF states is not instantaneous. Light has therefore become an attractive candidate due to its ease for external spatiotemporal control, rapid reversibility kinetics, and inexpensiveness. Müller et al. recently took an optogenetic approach by utilizing genetically encoded light switchable proteins to control cellular processes[34]. The group implemented a multi-chromatic system consisting of UVB, blue, and red/far red light and showed that three genes (angiopoietin-1 (Ang1), firefly luciferase (Fluc), and secreted embryonic alkaline phosphatase (SEAP)) could be individually induced in mammalian cells[34]. *In vitro* reconstitution of signals implicated in blood vessel formation was then attempted using human embryonic kidney (HEK) 293T cells containing blue-light controlled vascular endothelial growth factor (VEGF) and UVB-induced Ang1[34]. This experiment highlights the possibility to use the tools of synthetic biology to recreate processes that occur in nature. Building on this work, it should be possible to use different colors of light to study how interactions between multiple genes impact cell differentiation and tissue formation.

Optogenetic regulation has already ventured into tissue engineering-related applications. To assess cell migration within the context of an engineered microenvironment, Guo et al. transfected MSCs with Rac1 ─ a protein involved in actin polymerization[35]─that was fused to a light-activatable domain[36]. Cell encapsulation in poly(ethylene glycol) (PEG) hydrogels and subsequent stimulation at 458nm revealed that photoactivatable MSCs migrated linearly and with greater speed toward the light source than non-activatable controls[36]. Stem cell migration patterns were also evaluated both qualitatively and quantitatively using optogenetic regulation in combination with various parameters of hydrogel adhesivity, stiffness, or topography, providing a framework for examining the interplay between cells and biomaterials[36]. Another study relied on blue light pulses to spatiotemporally synchronize the contractions of Channelrhodopsin-2 (ChR2)-expressing murine myoblasts seeded in the wells of a miniaturized high-throughput device containing mechanical and chemical stimuli to generate 3D muscle microtissues[37]. This platform allowed for quantitative analysis of the engineered constructs as they developed, including mechanical stress, myoblast morphology, and fiber alignment[37]. The setup could be employed to monitor the formation of other tissue types and to elucidate the effects of different internal and external cues.

Another persisting issue that control at the molecular level could overcome is that of leakiness, whereby some residual gene expression occurs even when the device is maintained in the OFF state. Deans et al. constructed a tunable genetic switch such that in the absence of the chemical inducer isopropyl β-D-1-thiogalactopyranoside (IPTG), the LacI repressor inhibits transcription of the gene of interest as well as the tetracycline repressor protein TetR, which represses a short hairpin RNA (shRNA) targeted to the same gene of interest. Hence, the shRNA can freely bind to its complementary target sequence positioned after the gene of interest and tightly suppresses any leaky transcription of a reporter gene (enhanced green fluorescent protein, EGFP) by 99%[38]. In contrast, adding IPTG relieves LacI repression and activates TetR, which in turn halts shRNA transcription and strongly promotes expression of the gene of interest. In subsequent work, this gene regulatory device was transfected into mammalian cells that were then encapsulated in IPTG-containing scaffolds[39, 40]. This setup permits localized release of inducer molecules, which may be desirable if gene expression is to be initiated immediately. Further optimization of inducer release profiles

as a function of biomaterial degradation would be beneficial.

The exciting recent technologies of transcription activator-like effectors (TALEs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) provide customizable targeting specificity that could be used for analog and temporal multi-gene regulation in tissue engineering strategies. TALEs are proteins with transcriptional regulatory functions derived from the plant pathogen *Xanthomonas*[41]. Within each protein, a string of 34 amino acids organized in tandem repeats corresponds to individual nucleotides, and the twelfth and thirteenth residues—also known as repeat-variable di-residues (RVDs)—dictate the binding specificity to a target DNA sequence[42]. Konermann et al. designed a platform that merged TALE targeting specificity with optogenetic regulation. Transcription of a target gene resulted from the interaction of blue light-responsive proteins cryptochrome-2 (CRY2) and cryptochrome-interacting basic-helix-loop-helix (CIB1), which were fused to a TALE bound to a region upstream of a target gene and an activator protein VP64 respectively[43]. This method was used to upregulate expression of the Grm2 glutamate receptor in primary cortical neurons as well as in a murine model.

Projected to be more promising for programmable transcriptional regulation than TALEs, CRISPR interference (CRISPRi) is a relatively simple platform that relies on a catalytically inactive nuclease dCas9 derived from *Streptococcus pyogenes* and a short guide RNA (sgRNA) designed with sequence complementarity to the desired DNA binding site[44]. A complex formed by these two components hinders RNA polymerase binding and elongation, resulting in transcriptional repression of a targeted gene[45]. Though highly effective in bacteria, this strategy proved insufficiently robust in mammalian cells. Gilbert et al. therefore linked dCas9 to an effector protein that functioned either as an activator or repressor, and this modification demonstrated significant improvements in gene expression control[46]. Because only one protein and one sgRNA are required, and targeting specificity is easily encoded by simple nucleotide base pairing rules, CRISPRi technology is poised to be a tremendous asset for multiplexed gene regulation. In the meantime, CRISPRi can be used to precisely tune gene expression levels, thereby ensuring therapeutic outcomes and avoiding undesirable effects from over- or underexpression (Fig.1).
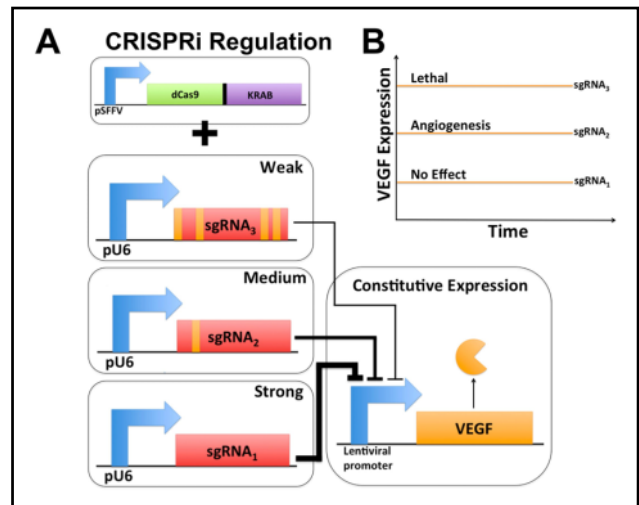

Fig.1 (A) CRISPRi technology can be used to precisely tune the expression levels of a gene of interest (ex. VEGF), introducing a layer of control that is otherwise not present when that gene is constitutively expressed. dCas9 is fused to a repressor protein (ex. Krueppel-associated box (KRAB)) that will inhibit transcription of the target gene[46]. Introducing mutations (orange bands) lowers sgRNA sequence affinity for its corresponding promoter and hence, decreases the level of gene repression. (B) Specific sgRNA sequences can be tested to identify appropriate levels of gene expression for therapeutic outcomes.

Toward complex behaviors

As described in the previous section, external control of gene expression using synthetic gene regulatory devices offers a systematic approach for improving current tissue engineering strategies, in addition to gaining a more comprehensive understanding of the cellular processes involved. A long-term goal of synthetic biology is to engineer complex cellular behaviors using collections of standard genetic parts[47]. To the extent that it achieves this goal, synthetic biology can therefore be used to further improve the degree of cellular control needed for tissue engineering applications.

One study described the development of a synthetic regulatory cascade, which linked three antibiotic-inducible expression systems (tetracycline, streptogramin, macrolide) in series to vary the percentage of gene expression depending on the administered drug[48]. This system would be applicable for stem cell reprogramming, as levels of a relevant transcription factor could be titrated to induce a

desired phenotype[48]. More recently, Ye et al. engineered a blue light-mediated signaling cascade — adapted from the phototransduction pathway used by melanopsin-expressing retinal ganglion cells—that was then used to control expression of glucagon-like peptide-1 (shGLP-1) in HEK 293 cells[49]. When these cells were implanted into normal and diabetic mice and stimulated with blue light, insulin levels could be successfully maintained by the shGLP-1 transgene, even following intraperitoneal glucose challenge[49]. The *in vivo* efficacy of an externally regulatable gene network extends the possibility of using a similar cascade for tissue engineering purposes.

Moreover, a number of biological analogues inspired by electrical circuits have been constructed from basic molecular parts. These systems are capable of behaviors including oscillations[50], time-delays[51], hysteresis[52], and band-pass filtering[53]. The characteristics of these circuits may be useful for programming different aspects of biological processes leading up to tissue development. Before such systems can be implemented, however, it is essential that all parts from the most basic level are modular and orthogonal (do not interfere with one another nor the host cell machinery), and that these devices can be predictably composed.

Cells can also be programmed with internal feedback for an additional layer of regulation. In one case, Burrill et al. transfected human cell lines with circuits containing feedback loops which would allow them to retain memory when subject to sufficient threshold concentrations of doxycycline, hypoxia, or UV exposure[54]. Memory of these stimuli was shown to propagate with subsequent cell generations, contributing to varied gene profiles, as well as affecting cell growth rates and viability[54]. This retention capability could be exploited in investigating the role environmental factors play in the differentiation of heterogeneous stem cell populations. More recent studies that explore *in vivo* outcomes suggest that the gap between synthetic biology and clinical translation is indeed narrowing. Kemmer et al. developed a synthetic circuit that autonomously sensed uric acid levels via mammalian-optimized hypothetical urase regulator (HucR) and its operator site hucO from *Deinococcus radiodurans*, which then led to concentration-dependent production of *Aspergillus flavus*-derived uricase responsible for solubilizing uric acid[55]. Delivering microencapsulated HeLa cells carrying this circuit into mice lacking urate oxidase led to decreases in both blood urate levels and uric

acid crystallization, which are associated with a number of human pathologies (ex. gout)[55]. Another self-sufficient circuit that relied on the concentration of fatty acids to activate pramlintide — a clinically approved hormone that decreases appetite — in a dose-dependent fashion was incorporated into human fibrosarcoma HT-1080 cells that were then encapsulated in alginate-poly(L-lysine)-alginate beads and implanted into murine models for obesity[56]. The outcome of this study was that blood lipid levels, appetite and body weight all decreased in the obese mice, whereas the circuit had no effect on control mice of normal size and diet[56]. Robust internal feedback networks such as these could be adapted for the reduction of inflammatory responses in damaged tissues *in vivo* by secreting therapeutic factors commensurate to the extent of injury.

Finally, a particularly exciting area that showcases network complexity involves Boolean logic, whereby cells can be made to integrate multiple input signals in order to control differentiation decisions. Ausländer et al. designed and combined various circuits so that cells could respond to inputs of erythromycin or phloretin, such that the following computations were possible: NOT, AND, NAND, and N-IMPLY[57]. These capabilities may further enhance the wiring of genetically engineered cells to their hosts *in vivo*, as they may be able to sense specific biomarkers from their surroundings, process these extracellular signals using combinations of logic gates, and accordingly express certain bioactive factors to promote local tissue regeneration. Hence, one might envision future treatment procedures involving the delivery of biomaterial-encapsulated cells harboring complex gene networks[26, 58] that can fluidly integrate with native tissues.

Conclusions and Future Perspectives

Despite tremendous advancements over the past decades, tissue engineering still faces significant challenges in generating constructs suitable for use in the clinical setting. Many of the strategies pursued rely on extracellular treatments with bioactive molecules, biomaterial-based controlled release technologies complicated by many parameters, and unregulated gene delivery—all of which have drawbacks. The molecular tools offered by synthetic biology provide an unprecedented level of genetic control, which would be invaluable for tissue engineering applications. External cell regulation allows for the systematic modulation of biological processes in a temporally relevant

manner, as well as elucidation of the mechanisms involved. Internal regulation using more complex circuits will eventually allow engineered cells to interface with the host environment and perhaps lead to better tissue integration[59]. Combining these two layers of control should improve cell-based approaches for tissue engineering and result in more predictable therapeutic outcomes[60]. In summary, we believe that collaborative efforts between tissue engineers and synthetic biologists are vital for accelerating the development and translation of effective regenerative medicine solutions.

Conflict of Interest

The authors declare no conflict of interest.

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