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Advances in Pluripotent Stem Cell Therapy: The Role of Synthetic Receptors and Genetic Circuits

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ABSTRACT

Pluripotent stem cells (PSCs) possess the unique capability to both differentiate into the primary three primary germ layers and self-renew, making them essential components for life's developmental processes. PSC-derived cells and multicellular structures, such as organoids, present significant opportunities for advancing regenerative medicine. However, progress in this area is still in its early phases, largely due to lack of effective methods for precisely controlling stem cell behavior that is highly controlled by internal network of genes responding to external environmental signals. Genetic circuits and synthetic receptors and genetic circuits emerged as valuable strategies for engineering cellular sensing and receptor mechanisms, with the potential to precisely guide cell fate and functional outcomes. This article reviews the current progress and key challenges facing PSC-based therapies and the creation of multicellular systems. Additionally, it highlights various synthetic biology tools used in PSC engineering and discuss the obstacles and future perspectives in applying synthetic biology to enhance regenerative medicine approaches

Keywords: Pluripotent stem cells, synthetic biology, stem cell engineering, regenerative medicine, organoids.

INTRODUCTION

Stem cell possess the remarkable capability to both differentiate into different cell types and self-renewal. During early development, they differentiate into precursor cells that eventually form complex organs. Depending on their capability to produce different types of cells, stem cells are categorized into four groups: totipotent, pluripotent (PSCs), multipotent, and unipotent. Totipotent stem cells, such as fertilized eggs and early-stage blastomeres, have the highest potential, able to develop into embryonic as well as extra-embryonic tissues[1]. Following a series of divisions, the totipotent cells give rise to the blastocyst, composed of the inner mass cell (ICM) and trophoblast. The ICM differentiates additionally into epiblast, which leads to PSCs, and the primitive endoderm. PSCs have the ability to become any cell types within the embryo but cannot form extra-embryonic structures [2]. Compared to totipotent cells, PSCs have a more restricted ability to differentiate, as they are limited to embryonic germ layers synthesis. Major advancements in stem cell exploration include the derivation of human embryonic stem cells (ESC) lines in 1998 from the blastocysts, and production of induced pluripotent stem cells (iPSC) from adult human fibroblasts in 2007 using specific transcription factors [3]. Both ESCs and iPSCs are classified as PSCs due to their limitless capability for self-renewal and their capability to differentiate into any cell type from the three germ layers. As PSCs undergo various stages of lineage commitment, they eventually give rise to multipotent stem cells, such as

hematopoietic stem cells (HSCs) that can differentiate into specific types of cells. In contrast, unipotent stem cells contain a very restricted differentiation capacity, being able to produce only one type of cell. Given these extraordinary properties, human pluripotent stem cells (hPSCS) possess pronounced potential in regenerative medicine, where the ultimate aim is the replacement or repair of damaged tissues, or even regenerate entire organs, restoring their full function [(4)].In cellular therapy, the stem cells with its derivatives' applications has been under investigation for various periods, though the field remains in its nascent stages. For acute myeloid leukemia (AM), the hematopoietic stem cell transplantation (HSCT) stands out as a well-established and effective therapy. In addition to HSCs, various stem cell types, including PSCs, have been explored in both pre-clinical and clinical contexts. Data indicates that there are currently, addressing a broad spectrum of conditions, such as musculoskeletal disorders, neurological diseases, and COVID-19-related lung issues. However, the majority of PSC-based therapies remain in pre-clinical stage, with approximately 130 clinical trials investigating treatments for diseases like Parkinson's disease, macular degeneration, spinal cord injuries, heart failure, and corneal disorders. Several challenges impede the clinical implementation of PSC-based therapies, including issues with immune rejection, potential tumor formation, difficulties in achieving consistent and mature differentiation of specific cell types, and challenges related to scaling up cell production while maintaining quality control. Addressing these issues is crucial for advancing the field.

Beyond their role in cell therapy, human PSCs have also been utilized to create complex multicellular tissues *in vitro*, with the aim of developing organ or tissue-level constructs for future regeneration and repair. Advances in technologies of stem cells, e.g., synthetic and organoid embryo technologies, have enabled the creation of intricate multicellular systems that replicate early embryonic development. By employing tailored small-molecule mixtures to simulate developmental pathways *in vivo*. Organoids derived from PSCs have been successfully generated for various organs. Recent research has also led to the development of synthetic blastoids of human, which model early development and implantation of blastocyst. Nonetheless, further refinement is needed to meet clinical transplantation criteria, such as achieving optimal functionality, appropriate tissue size, improved immune tolerance, reduced costs, and enhanced reproducibility. To expedite advancement in PSC-based regenerative medicine, interdisciplinary collaboration is essential, integrating development in 3D bioprinting, systems biology, and the synthetic biology. Unlike customary top-down methods for studying developing processes and regulating cell functions, synthetic biology offers a bottom-up approach that enables precise, programmable regulator of cell behaviors. Originally discovered in yeast and bacteria because of their simpler genetic networks, synthetic biology gained prominence with the creation of the first *Escherichia coli* bistable switch in in 2000, which allowed for controlled substituting among two stable states of gene expression. Likewise, a genetic oscillator with a feedback loop was designed, achieving episodic repressor protein expression oscillation [(5–11)]. These milestones highlighted the onset of synthetic biology as a distinct field.

Further progress was made with the development of genetic circuits using transcriptional regulators and promoters that functioned as binary logic gates, facilitating complex Boolean logic computations in the cells. Over time, more sophisticated genetic circuits, including band-pass counters and filters, have been established. With rapid advancements in gene editing and a deeper understanding of cell biology, synthetic biology has expanded from bacteria and yeast to include mammalian cells. This expansion underscores the potential of synthetic biology to accurately control the PSC and its derivatives' functions. This review is focused on the crucial role of synthetic biology in the bioengineering of PSCs for advancing regenerative medicine. The review explores the PCS's applications in this domain, address the limitations that need to be overcome, introduce key tools of synthetic biology used in the mammalian cells, and demonstrate how these tools are driving the growth of regenerative therapies based on PSCs. Finally, we deliberate the challenges and future viewpoints of PSC engineering with synthetic biology for therapeutic purposes.

2. Applications in Regenerative Medicine

Owing to exceptional qualities of the hPSCs highlighted, they are viewed as a highly suitable foundation for application in regenerative

medicine. hPSCs hold the capability to produce an extensive range of cellular types for utilization in cell-based treatments, as well as to construct complex multicellular systems that could eventually be utilized for organ transplantation.

2.1. Cell Therapies

The hPSCs and their derivatives have been explored as potential treatments for various difficult-to-treat diseases, with many hPSC-based products now advancing through preclinical and clinical studies. A significant early clinical trial for hPSC-based therapy took place in 2010, utilizing hESC-derived oligodendrocyte progenitor cells for treatment of spinal cord injury (SCI) [12]. Two other clinical trials were initiated to treat Stargardt's macular dystrophy and dry age-related macular degeneration using hESC-derived retinal pigment epithelium (RPE) cells, demonstrating survival and safety of transplanted cells, with indications of biological activity [13]. In 2014, the first iPSC-based clinical trial was launched, targeting eye diseases. The trial involved the injection of RPE cells derived from iPSCs into a patient with macular degeneration [14]. However, it was halted due to concerns about tumorigenicity related to two genetic mutations.

The hPSCs are also investigated for cardiac disease therapies. In a phase I ESCORT trial, the safety of hPSC-derived cardiac progenitor cells was tested in 10 patients having severe heart failure, using fibrin gel scaffolds to deliver the cells [15]. The trial demonstrated that it was feasible to generate cardiomyocyte progenitors derived from hESCs. No adverse events linked to the cells or scaffolds were seen, even with follow-up extending up to six years. More recently, another trial investigated the safety of cardiomyocytes derived from iPSCs entrenched in cell sheet for the transplantation.

In the field of neurological disorders, hPSC-derived dopaminergic neuron replacement has been prominent therapy, particularly in Parkinson's disease (PD). The first phase I/IIa clinical study using neural precursor cells derived from hESCs for PD focused on the safety and efficacy of these cells when transplanted into the brain [16]. iPSC-based clinical research covers conditions like cancers, heart failure, graft-versus-host disease, macular degeneration, PD, and corneal disorders. Research involving hESC-derived cells focuses on conditions such as Parkinson's macular degeneration, intrauterine adhesions, type 1 diabetes, and amyotrophic lateral sclerosis.

Despite the progress in PSC-based clinical trials, several key challenges remain, including risk of immune rejection, tumor formation, achieving reliable and consistent differentiation of particular cellular types, which can survive proliferation, and perform appropriately, and scaling up production while maintaining quality control.

A significant hurdle that needs to be overcome before the broader clinical utilization of PSCs and their derivatives is the issue of immunogenicity. The immune rejection remains a main barrier for the allogeneic cell transplants, primarily resulting from human leukocyte antigen (HLA) mismatches between donor and recipient cells. Besides HLA mismatches, other factor contribute to this problem, including the expression of immune antigens because of sustained culture *in vitro*, abnormal antigen production from genomic mutations, inadequate somatic cell reprogramming during iPSC generation, and the PSC-derived cells' immature immunity [17]. While autologous cells derived from iPSCs, tailored to individual patients, may sidestep these issues, they tend to be prohibitively expensive due to rigorous quality control requirements and are often too slow to address urgent medical needs. A potential solution lies in the establishment of HLA-matched iPSC banks, which could deliver standardized cell sources for the clinical applications, resolving both price and scalability problems. Another innovative method involved HLA cloacking, where HLA genes are deactivated or immunosuppressive factors are overexpressed in allogeneic stem cells [18]. This reduces the cellular immunogenicity, making them more appropriate for large-scale, standard therapeutic use. With such strategies, a limited hPSC lines, or even a solitary universal line, could function as an allogeneic cell source for the global population. These universal hPSCs would offer greater homogeneity, lower costs, and improved safety, making them promising options for engineered, standard cell therapies that can be applied on a large scale.

The self-renewal ability of hPSCs, though crucial for their functionality, also presents a challenge due to the risk of uncontrolled cell proliferation, which can lead to tumorigenesis following transplantation. Moreover, progenitor cells used in cell therapies have also demonstrated tumorigenic tendencies after being transplanted. Tumor development can be triggered by various

factors, comprising cells having unusually great proliferation rates, genetic aberrations arising from extended culture, or alterations caused by gene-editing techniques. A recent study highlighted that above 20% of the hPSCs and their derivatives contained mutations like malignancy, with majority of them involving TP53 gene, a key tumor suppressor that is commonly associated with cancer progression when mutated [19]. This underscores the critical need for thorough monitoring of hPSCs for oncogenic mutations, particularly when considering their use in clinical settings. Although the complete elimination of tumorigenic potential in hPSCs and their derivatives through genetic engineering remains a challenge, proactive strategies are essential. One effective method involves ongoing surveillance and removal of cells exhibiting tumorigenesis traits. For instance, stem cells can be equipped with inducible elimination or suicide mechanisms, e.g., fail-safe systems depending on suicide genes like HSVtk and Caspase9, or switches controlled by miRNA [20,21]. These approaches offer a way to selectively eliminate problematic cells, enhancing the safety of regenerative therapies.

Differentiating PSCs into target cell types is essential for developing cell-based therapies. However, this process encounters several challenges, such as inefficiencies and inconsistencies that lead to cells having poor purity, undeveloped functionality, and variable reproducibility. Effective cellular replacement treatment requires that transplanted cells not only survive and proliferate but also accurately migrate to and integrate within the therapeutic site, where they must perform their designated functions. A significant issue is the difficulty that donor cells face in establishing functional interactions and integration with the recipient tissues. For example, in trials involving RPE cells derived from PSCs for treating age-related macular degeneration (AMD), cells often failed to correctly position at the intended place. Similarly, in PD therapies using PSC-derived dopaminergic progenitor cells, challenges such as maintaining cell durability, ensuring survival, achieving effective synaptic integration, and restoring function after transplantation persist [22].

The production of hPSCs and their derivatives, like other biologics, must follow rigorous good manufacturing practice (GMP) standards to ensure safety, effectiveness, and viability of these cells for large-scale applications. The natural variability of hPSCs poses challenges, including inconsistencies among clone lines, variations within cells of the same clone during prolonged culture, and changes in gene and protein expression levels over short period. Additionally, long-term cultivation of hPSCs can result in genetic mutations, and their differentiation *in vitro* may lead to diverse cell subpopulations [17]. Therefore, it is essential to develop efficient and less labor-demanding strategies for controlling the pureness and genetic consistency of the hPSCs to maintain safety of product. Recent advancements in bioreactor technology have provided a cost-effective solution to overcome the difficulties of traditional, labor-intensive production methods [23].

The process of differentiating PSCs to specific types of cells is a fundamental phase in developing therapies based on these cells. This is typically achieved by sequentially adding signaling inhibitors, growth factors, and small molecules to mimic growing conditions in extracellular environment. Though, this process faces major hurdles, such as low efficiency and variability, which can result in the production of immature cells or those with tumorigenic potential. Inconsistencies in differentiation can lead to variability in the therapeutic potency and function of the resulting cells. For example, hESC-derived cardiomyocytes may express key marker transcription factors and show cardiomyocyte-like characteristics but still exhibit immature electrical behaviors [22]. Reliable and efficient PSC differentiation protocols are essential for ensuring therapeutic efficacy. To address these issues, researchers are exploring improved culture conditions and genetic engineering techniques, aimed at promoting cell differentiation and maturation. Studies have demonstrated that optimizing timing, dosage, and combination of signaling inhibitors, growth factors, and small molecules can enhance the differentiation effectiveness and reliability[24,25]. Other approaches include overexpressing key transcription factors to accelerate cell maturation. In addition, innovative culture platforms like 3D bioreactors and microfluidic maneuvers that better stimulate normal environments are helping to improve control over the differentiation process [23]. A more advanced strategy involves gaining deeper insights into networks of genetic regulation controlling the differentiation of stem cells *in vivo* and using this knowledge to guide *in vitro* differentiation [26]. Emerging analytical tools that integrate gene expression data, proteomics,

and cell signaling pathways are being developed to identify critical transitions during the differentiation process.

3. Advancements in PSC-Derived Systems

The creation of multicellular systems that mimics the physiological utilities of tissues or organs *in vitro* represents a pivotal and highly promising use of PSCs and their derivatives in regenerative medicine approaches. The fundamental principle behind these systems lies in the cells' innate ability to self-organize. Building to this discovery, scientists have made significant strides in assembling complex tissues and organs using dissociation and reaggregation methods. A landmark achievement came with the effective development of the 3D mini-intestinal tissue featuring structures of crypt-villus, derived from individual stem cells (Lgr5+) [27]. Since then, numerous approaches have been refined to make human organoids, which are small-scale models of organs or tissues that replicate physiological structures and utilities *in vitro*. These organoids, derived from both stem cells and PSCs in adults, include intestinal organoids, optic cup organoids, and others such as cardiac, cerebral, pancreatic, and kidney organoids [5,6,8,10,28,29].

To further upsurge the organoids' complexity in terms of cell diversity and functionality, scientists have merged various cell types or organoids to make assembloids. For example, corticostriatal assembloids exhibit interactive neural connections, and combining striatal and cerebral cortical organoids has led to the development of midbrain-microglia assembloids, which show enhanced neuronal development and metabolic activity [30]. In addition, advanced technologies such as 3D bioprinting have been integrated to refine organoids features [31]. This approach has been particularly useful in producing vascularized organoids, such as gas-exchanging lung organoids, and in generating highly uniform kidney organoids suitable for high-throughput production. Despite these innovation, hPSC-derived organoids face several challenges that limit their application in regenerative medicine.

While organoids demonstrate better structural organization compared to 2D monolayer cultures, they are still unable to fully replicate the complex spatiotemporal architecture of organs. One contributing factor to this limitation is the absence of key lineage cells, such as stromal or vascular endothelial cells. Additionally, the *in vitro* environment lacks the necessary morphogen gradients that are vital for guiding tissue organization during *in vivo* development. Strategies like cell engineering, 3D bioprinting and other advanced techniques offer potential for enhancing structural integrity of organoids by addressing these challenges. Organoids offer a significant advantage due to their upgraded tissue-like functionality, effectively overcome the gap between the limitations of 2D monolayer cells and animal models. However, most organoids derived from hPSCs tend to show fetal-like cell types and functions, as revealed by multi-omics analyses. For example, liver organoids show fetal hepatocyte features, i.e., cytochrome P459 activity, inflammatory response, storage of vitamin A, and accumulation of lipids. Likewise, cerebral cortical organoids mimic the brain development at 19 to 24 weeks post-conception [32,33]. Few studies have achieved the creation of organoids derived from PSCs with neonatal-level functions. The hypothalamic arcuate organoids were produced in 2021, displaying cellular diversity and molecular characteristics similar to neonatal human hypothalamus, although their neonatal-level functionality was not demonstrated [34]. Overcoming these challenges may require refining differentiation protocols, integrating additional cell lineages, and reconstructing the microenvironment.

The challenges of organoid research also include their restricted size and lifespan size, which are linked but distinct issues. These problems mainly stem from inadequate supply of nutrition and inefficient removal of wastes as organoids grow. As the size of organoids increases, diffusion becomes less effective, leading to restricted development, cellular death, and eventual necrotic death. To combat such limitations, scientists have established approaches like utilizing bigger bioreactors, incorporating perfusion systems to advance access of nutrition and removal of wastes, and enhancing vascularization in order to better distribute nutrients via capillary-like networks [23,35]. To advance the use of PSC-derived organoids for organ or tissue replacement, there is a need for the development of cutting-edge technologies and interdisciplinary methods to resolve the current challenges. Beyond organoids, another multicellular systems known as synthetic embryos has emerged, including human blastoids. In 2021, multiple research groups

stated the successful human blastoids creation, which were produced either through ESC aggregation, naïve ESCs' self-organization, or fibroblast reprogramming. One research group also developed blastoids derived from naïve PSCs that mimic blastocysts such as epiblast, primitive endoderm, and trophectoderm, and attached them to endometrial cells having polar trophectroderm, modeling the imbedding process [35–37]. Such synthetic embryos deliver valuable simulations for studying development, disease mechanisms, *in vitro* organ regeneration, and drug testing.

4. Utilizing Synthetic Biology to Engineer PSCs

Synthetic biology offers a framework to re-engineer or create cellular behaviors by utilizing three fundamental modules i.e., signal detection, and cellular response. Cells can detect various external or internal signals through synthetic receptors or sensing molecules. These signals are processes via natural cellular regulatory systems or artificially designed genetic circuits, which then lead to specific cellular responses. In synthetic biology, tools such as devices and circuits capable of sensing diverse signals, such as extracellular inputs, and generating a custom output like a transcriptional response, are critical for regulating cellular functions [38]. Recent advancements in programmable, modular receptor system allows scientists to alter the input-output dynamics of cells. These synthetic receptors, which can form new input-output relationships, have broad applications ranging from cellular therapies to the development of multicellular systems.

Natural receptors in mammalian cells play a key role in sensing, processing, and responding to environmental stimuli. By leveraging knowledge of these natural systems, synthetic biology has allowed the improvement of engineered receptors that can detect customized signals and generate use-defined responses. When combined with processing tools, these synthetic receptors allow for controlled manipulation of cellular behavior. To enhance and expand synthetic receptor designs, they are typically divided into two main parts, i.e., the intracellular actuator and the extracellular sensor. The sensor is responsible for recognizing environmental cues, whether natural or engineered, while the actuator translates these signals into a response through either natural signaling pathways or custom genetic programs [38]. These compounds both natural and synthetic, can be modularly integrated.

A well-known example of synthetic receptor and an engineered sensor is chimeric antigen receptor (CAR), which modifies T cells signaling pathways by replacing the natural ligand-binding domain with an antibody-based domain, while preserving specific surface antigens [39]. Other synthetic receptors, like generalized extracellular molecule sensors (GEMS), use synthetic sensors with natural signaling to activate endogenous pathways [22]. Systems like Tango and ChaCha employ natural sensors combined with synthetic actuators to induce specific responses into internal signals. Fully synthetic receptors, i.e., modular extracellular signaling architectures (MESA), synthetic Notch receptors (synNotch), and synthetic intramembrane proteolysis receptors (SNIPRs), offer refined control over both input detection and output generation. The final step in controlling behavior involves transcriptional signaling processing, which connects receptor activation to gene expression [40–43]. By designing or modifying transcriptional regulation systems, researchers can regulate the gene expression in response to signals received by either natural or synthetic receptors, offering precise regulation over cellular functions.

5. Designing PSCs Using Synthetic Biology

hPSCs offer a significant advantage in cell therapy because of their capability of differentiation into a diverse types of cells, including those that are challenging to get from human tissues or have limited growth potential *in vitro*. To refine the regulation of gene product levels, synthetic lineage-controlling circuits presents a promising solution [44]. These circuits can precisely guide cell fate decisions, enhancing the efficiency and purity of the desired cell types, which could facilitate their use in clinical settings and large-scale production. Research indicates that synthetic genetic circuits could be a powerful tool for generating cells derived from hPSCs by directly managing the expression of critical developmental genes. For instance, a synthetic lineage-control network inspired by pancreatic development in mice has been developed. This network replicates the timing patterns of important transcription factors such as pancreatic and duodenal homeobox 1 (PDX1), V-maf

musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA), and neurogenin 3 (NGN3). By employing a signaling cascade and a gene switch, this network enables the precise timing of these factors' expression in hiPSC-derived pancreatic progenitor cells. The β-like cells produced show glucose-sensitive insulin secretion akin to human pancreatic islets, highlight the probability of synthetic gene circuits to create functional cells or therapeutic use.

Despite of these advances, many hPSC-derived cells still encounter issues such as underdeveloped functionality, high variability and low efficiency when using current differentiation techniques involving small molecular cocktails and growth factor inhibitors. Forward programming approaches, which involve the forced transcription factors (TFs) expression, provide a latent solution. For instance, thyroid follicular cells and transplantable organoids have been created from human and mouse PSCs via transient overexpression of paired box gene 8 (PAX8) and NK2 homoebox 1 (NKX2-1). Additionally, robust differentiation of functional thyroid progenitors from anterior foregut endoderm derived from mouse PSCs has been achieved through transient NKX2-1 overexpression at specific developmental stages. Other studies have produced megakaryocytes from hPSCs using TAL1, GATA1, and FLI1 overexpression, and differentiated hPSCs into oligodendrocytes, neurons, and functional skeletal myocytes. However, these expression methods still face challenges in precisely controlling cell fates due to difficulties in managing the spatial and temporal aspects of gene expression [44].

A major challenge in differentiating PSCs is the creation of a heterogeneous cell population, which complicates the purification and detection of certain types of cells needed for treatment. To tackle this issue, synthetic cell classifiers utilizing microRNA (miRNA) switches have been developed to effectively identify and isolate the target cells. In 2015, Miki introduced these synthetic miRNA switches specifically designed for isolating cell types during hPSC differentiation [45]. These switches include miR-499a-5p, miR-1, and miR-208a for cardiomyocytes, miR-122-5p for hepatocytes, miR-375 for insulin-producing cells, and miR-126 for endothelial cells. For example, miR-1 switch uses synthetic mRNA to produce a fluorescent protein (FP) having a miR-1 target sequence. Cells that naturally express miR-1 will not produce this FP. Morever, miR-1-Bim switch incorporates apoptosis-induced protein Bim, which triggers cell death specifically in cells that do not express miR-1, thereby eliminating non-target cells [45].

To advance therapeutic applications beyond basic physiological functions, significant improvements can be made, i.e., targeting and destroying cancer cells. Achieving consistent and accurate treatment outcomes necessitates either reengineering existing cellular networks or creating fresh ones. The synthetic biology offers a valuable tool for enhancing cell-based treatments by allowing accurate regulation of context, timing, and intensity of treatment actions. Cells can be designed to detect specific signals, including small molecular and biomarkers of disease, and subsequently control localization, dosage, and timing of therapeutic gene expression or other functions. A major landmark in the arena was the Tisagenlecleucel, a CAR T cell therapy for treating resistant pre-B cell ALL and diffuse large B cell lymphoma (DLBCL) [33]. This treatment includes amending T cells of patient to prompt chimeric antigen receptors (CARs), which precisely target tumor cells. Despite its potential, challenges, such as limited cell availability, high costs, and engineering difficulties limits its widespread use. Integrating PSC differentiation technologies with synthetic CARs may address these issues. A significant development in this area is the creation of CAR iPSC-derived NK cells, which offer a safer, more standardized and readily accessible option for cancer treatment [46]. Early trials have demonstrated that iPSC-derived NK cells are both effective and safe. These cells, initially derived from hESCs, have protocols that are now adapted for use with human iPSCs. Engineered NK cells derived from PSCs deliver a continuous and constant source of NK cells, eliminating the need for individual donor or cord blood collections. Additionally, genetic modifications are more easily introduced into PSCs compared to primary NK cells, enabling the integration of multiple enhancements, such as CARs, to improve cell functionality [47,48]. The long-term achievement of these therapies rest on the persistence of cells *in vivo*. Genetic modifications like overexpression of IL-15 or knockout of CISH can promote cell development, although these changes may affect the process of differentiation from PSCs to mature types of cells [49]. Future approaches may involve inducible gene editing to address these challenges. By employing genetic

circuits with CARs and modifications induced by food additives, PSCs can be engineered to produce NK cells with improved persistence. Additionally, engineered cells equipped by prostate-specific antigen (PSA)-specific GEMS have been developed for detecting abnormal levels of PSA in serum of prostate cancer patients, aiding in diagnosis [50].

A significant issue with employing hPSCs in cell therapy is the risk of tumorigenesis associated with residual undifferentiated PSCs and progenitor cells. To enhance treatment safety, it is essential to continuously monitor and remove these potentially tumorigenic cells. One effective approach involves engineering cells having inducible elimination or suicide switches, allowing for real-time control and removal of any tumorigenic risks. Recent studies have shown that miRNA switches can successfully detect and eradicate undifferentiated cells in the iPSC-derived midbrain dopaminergic (mDA) neuronal cells [21]. For instance, the miRNA-302a-5p (miR-302a) switch was engineered to exploit its high expression in PSCs. This switch activates a reported protein when iPSCs differentiate into mDNA cells and miR-302a level decrease. In SCIS mice, mDNA cells with this switch, when combined with iPSCs, did not lead to teratoma formation. Additionally, a puromycin-resistant gene regulated by the miR-302a switch allows for selective *in vitro* elimination of iPSCs using puromycin. The neural stem/progenitor cells (NS/PCs) derived from iPSCs have latency for treating spinal cord injuries (SCI), but their use has been linked to tumorigenesis in NOD/SCOD mouse models. To solve this probkem, scientists have developed fail-safe systems utilizing suicide genes such as HSVtk and Caspase9 [20,51]. In NOD/SCID mice, the small molecular CID induces Caspase9 expression, causing rapid apoptosis of all transplanted cells in the injured spinal cord. The HSVtk/GCV system, on the other hand, targets actively dividing tumorigenic cells by converting ganciclovir (GCV) into a cytotoxic form through HSVtk. This system has been effective in preventing tumorigenesis and preserving mature neural cells, thereby supporting motor function improvement in SCI models.

6. Utilizing Synthetic Biology to Develop Multicellular Systems from PSCs

6.1. Cell-to-Cell Communication

Synthetic biology enables the creation of artificial intracellular communications systems, wither through direct or paracrine signaling. One of the most effective tools for this purpose it the synNotch system, a synthetic receptor engineered to control interactions between cells. This system consists of a sender cell that presents a specific surface antigen and a receiver cell equipped with a synNotch receptor [42]. The receptor includes an extracellular domain (ECD), transmembrane domain (TMD), and an intracellular transcription factor domain. Upon contact between the cells, the receptor detects the antigen, causing the release of the transcription factor and activating target gene expression. The synNotch system has a wide range of uses, including organizing cell spatial patterns and tracking organoid differentiation. By combining different synNotch receptors, concentric arrangements of cells can be created in both 2D and 3D structures [42,52]. This system has also been utilized for lineage tracing and monitoring cell interactions during embryonic development in mice [53]. Moreover, synNotch can be engineered to influence cell differentiation pathways, such as triggering neuronal differentiation via Neurogennin 1 expression, or directing the production of morphogens and adhesion molecules to control cell positioning and organization [54,55].

6.2. Morphogen Signaling

In developing organs, the spatial and temporal patterns of cell positioning and fate are directed by gradients of signaling molecules known as morphogens. Cells adjust their gene regulatory networks (GRNs) in reaction to varying morphogen levels, ultimately determining their fate and forming organized multicellular structures. This concept has led to efforts in engineering stem cells to replicate morphogen-derived signaling or GRNs, potentially enabling the synthesis of complex multicellular systems. To mimic how morphogen gradient control cell behavior, optogenetic chimeric receptors have been designed. These receptors, which fuse a light-sensitive domain with the cytoplasmic part of morphogen receptor, allow for precise manipulation of pathways like Wnt, BMP, TGF-β, ERK, and FGF within mammalian cells [56–58]. An example includes the use of chimeric receptor combining the blue-light-responsive Cry2 domain with the cytoplasmic region of LRP6, which successfully stimulated Wnt signaling in a subset

of hESCs [56]. This approach induced mesoderm differentiation and spatial self-organization, highlighting the potential of synthetic receptors to replicate morphogen signaling for creating multicellular systems.

Beyond controlling morphogen-driven signaling, the recreation of morphogen gradients can facilitate the spatial and temporal precision needed for multicellular system construction [54]. A modified synNotch system was used to create an orthogonal synthetic morphogen system that detects soluble GFP secreted by surrounding cells, establishing a GDP gradient using anchor and receiver cells. While GFP was used as a mode, the system can be adapted for various morphogens with appropriate anchors. This study demonstrates the feasibility of generating multicellular systems through synthetic receptor technology, offering broader potential applications in tissue engineering and developing biology.

6.3. Cell Adhesion

The formation of multicellular systems *in vitro* often depends on the self-assembly of cells, which can result in considerable variability in both cell types and shape due to random aggregation, unlike the tightly regulated processes observed *in vivo*. To address this issue, controlling cell-cell adhesion artificially could be a crucial strategy for developing tissue-like structures with more precise architectures. Both natural and engineered cell adhesion systems, such as synNotch-cadherin, synCAM, and helixCAM, have been created [52,59,60]. The system of synNotch-cadherin has been successfully implemented in chimeric embryos and mouse ESCs, demonstrating potential for use in human PSC-derived cells for the artificial tissue assembly. Such adhesion systems show promise in improving embryoid formation. For instance, altering the expression of cellular adhesion molecules are found to enhance the competence of embryoid assembly by up to threefold. These embyoids were generated from a mixture of ESCs, extraembryonic endoderm cells, and trophoblast stem cells [61]. Employing these cell adhesion tools can enhance self-aggregation and support the development of more structured, organ-like formations.

6.4. Lineage Differentiation

Development is precisely managed by intracellular gene regulatory networks (GRNs), which response to external signals and internal pathways. Current techniques for creating organoids with multiple cell types typically involve either combining different cell types or applying small-molecule cocktails to drive simultaneous differentiation. These approaches are limited by the difficulty of developing protocols for diverse multi-lineage differentiation and the need for appropriate culture media to support various cell types. Recent advancements in omics data, predominantly profiles of single-cell transcriptomics from different organs and tissues, provide a chance to compare the expression of gene among organoids derived from hPSCs and their counterparts *in vivo*. This comparison can guide the creation of synthetic genetic circuits to more effectively control multi-lineage differentiation, causing organoids having improved structural and functional properties. For example, a proof-of-concept study successfully used genetic circuits to upregulate ATG5 and PROX1 to activate endogenous CYP3A4 via CRISPR technology, generating human liver organoids with endothelial cells, stellate cells, hepatocytes, and stellate cells [26].

7. Challenges and Future Perspectives

The discovery of hPSCs has sparked optimism for progress in regenerative medicine, particularly in the utilization of PSCs for cellular replacement therapies and the creation of multicellular systems. Nevertheless, the widespread clinical adoption of hPSC-based therapies is still hindered by several key barriers. These include the limited effectiveness of existing differentiation protocols for producing specialized cell types or organoids, the immunogenic challenges posed by hESCs in allogeneic treatments, and the tumorigenic risk associated with both hPSCs and their progenitor cells. As previously mentioned, leveraging synthetic biology techniques to modify PSCs presents promising solutions to address these challenges.

A deep understanding of GRNs specific to various cell types is crucial for precisely controlling cell fate and state, and these networks offer key insights for advancing stem cell engineering. However, many GRNs linked to cell fate determination and function reconstruction in specific cell types remain largely unexplored. Recent progress in systems biology and artificial

intelligence (AI) has led to the creation of models that manipulate single or multiple genes, helping to predict how these genes influence transcriptional outcomes. By incorporating these data-driven methods into stem cell engineering, we can gain a more comprehensive understanding of gene functions and GRNs, forming the basis for targeted manipulation of stem cells. Omics data, for instance, can be used to track changes in cell states through systemic experimentation or with AI-powered models. These approaches can uncover the critical GRNs and TFs responsible for regulating these transitions. An example is the 2023 construction of a TF atlas, which systemically screened TFs in ESCs by overexpressing them using a ORF library that included various spicing variants of all TFs in human, significantly advancing our knowledge of GRNs [62]. Additionally, machine learning tools, such as cSTAR, enable the integration of omics facts to model the mechanistic systems that drive cellular state changes [63]. Such breakthroughs pave the way for designing genetic circuits that can precisely steer and manage stem cell fate. Beyond understanding the fundamental principles that guide the behavior of individual PSCs, it is essential to explore how the cooperative actions of these cells lead to complex multicellular dynamics and self-organizing phenomena, including symmetry breaking and formation of pattern. Further studies are necessary to determine how diverse cell types can be spatially arranged and how boundary conditions can be established *in vitro*, including considerations such as the composition of ECM and the overall geometry of developing tissues or organs.

Several synthetic genetic circuits have been developed to manage input, output, and processing mechanisms. Though, some developmental components remain challenging to control using synthetic biology approaches. One such component is the ECM, which is crucial for cell signaling. Once formed and altered by the sender cells, the ECM conveys signals to the receiver cells via its molecular composition and mechanical characteristics. As its size is large and structure is complex, regulating ECM production, modification, and signal transmission remains difficult, although these functions are vital for organoid development. Recently, an algorithm of machine learning called Protein MPNN was utilized to create new fibrous proteins [64]. By adjusting the pore size of these artificial fibers, their mechanical properties can be fine-tuned, with smaller pores producing stiffer fibers. This level of control is particularly valuable for protein-based hydrogels, where fiber porosity can be manipulated to alter the bulk modulus, offering promising tissue engineering. Tolls of synthetic biology offer a distinctive chance to develop designer stem cells having closed-loop circuits capable of monitoring and responding to diseases in real time. These engineered stem cells are designed to detect environmental signals and release specific molecules that influence cell fate, function, and behavior. Incorporating closed-loop circuits into stem cells could allow for precise therapeutic interventions, enabling personalized treatment that adapts to the patient's condition as it evolves. One latent use of this tool is in tumor therapy, where immune cells derived from PSCs can be engineered for expression of receptors that recognize precise biomarkers of cancer. Upon detection, a signaling cascade triggers the therapeutic agent secretion like drugs, cytokines, or chemokines, which precisely target the tumor cells while diminishing damage to healthy cells. Another promising use is in diabetes management. Scientists have created β-cell-like designer cells by engineering of HEK-293 cells having a closed-loop system that links glycolysis-induced entry of calcium to a transcription mechanism that triggers GLP-1 or insulin production [65]. This system allows the cells to continuously monitor glucose levels and secrete insulin or supplementary controlling molecules in response. Furthermore, such circuit can be improved to release cytokines or growth factors, promoting regeneration of pancreatic cells, and modulating immunological responses to increase control of blood sugar. In the upcoming, β-cells derived from PSCs with advanced closed-loop systems could provide continuous monitoring and treatment for diabetes.

Synthetic gene circuits, which allow for the monitoring and detection of disease-related molecular changes, embrace abundant potential for improving the accuracy and rapidity of phenotype-based drug discovery (PDD). This approach can be applied across various cellular systems, such as cells and organoids derived from hPSCs [66]. Nevertheless, a key obstacle is the challenge of designing new circuits predictively from the ground up. AI has the potential of aid in the automated development of effective gene circuits, which can monitor specific biological phenotypes and mechanisms. In upcoming applications, for instance, engineering

neurons derived from hPSCs with advanced genetic circuits could allow for the detection and monitoring of broad neurodegenerative disease features. This might include tracking protein aggregate formation with a sophisticated reported circuit and identifying hyperexcitability using a frequency-decoding circuit, thus greatly enhancing drug discovery processes. Synthetic biology is set to revolutionize stem cell engineering by improving the precision, orthogonality, and flexibility of tis tools. Unlike the straightforward genetic networks in mammalian cells, bacteria, and yeast, have more intricate GRNs. Specificity of the synthetic biology implements comes from their capability to target and modify distinct physiological mechanisms without distressing others, attained via miRNA, specific ligands, or TFs that precisely attach to their targets. Synthetic biology orthogonality means that various circuits of genes can function independently without interfering each other. Achieving this requires meticulous controlling and modification of each circuit constituent. This include using orthogonal genetic operators to ensure that circuits operate independently in space and time, selecting orthogonal regulatory elements to create functional networks between circuits, and choosing suitable expression vectors and cell systems to manage gene circuit expression and cell viability effectively. Furthermore, engineering synthetic circuits for different cell types during PSC differentiation presents challenges. Genetic components, especially those involved in transcriptional regulation, can behave inconsistently across various cell types due to their partial overlap with endogenous GRNs. Additionally, there is the issue of cellular burden associated with integrating genetic circuits into mammalian cells. The introduction of these circuits can cause unintended interactions with the cell's own gene expression systems because of inadequate cell resources. Such issue might be addressed through co-expression of miRNAs and RNA-binding proteins, representing that a carefully designed and optimized approach to genetic circuits could enhance cell function while managing cellular stress and maintaining functional outputs [67].

These toolkits, mainly developed for bacteria and yeast, are instrumental in controlling cellular functions and engineering metabolic networks. However, their effectiveness is limited in mammalian cells, underscoring the need for specialized toolkits for stem cell engineering. This involves creating natural molecule-based toolkits as well as designing novel artificial sequences of DNA or protein domains. Recent advancements in synthetic biology had demonstrated significant progress in extending the lifespan of yeast cells. A study successfully altered a natural toggle switch to establish genetic clock, using lysine deacetylase Sir2 and a heme-activated protein (HAP) to regulated aging processes between nucleolar and mitochondrial systems. This modification has the potential to delay cellular aging and could lead to breakthroughs in the regenerative medicine and treatments for age-linked conditions [68]. Adapting these techniques for human cells will required further research. In synthetic multicellular systems, emerging tools are enabling a wide range of developmental processes. Developments such as TFs, multi-input logic states, and additional genetic components are creating versatile lineage-control networks. For instance, the MultiFate synthetic gene circuit can generate multiple stable states within a single mammalian cell [69]. Such system utilizes programmable zinc finer TFs that can do self-activation as homodimers and prevent each other when making heterdimers, regulated by small molecules. With expressing three distinct TFs, a single cell life can achieve seven diverse states of cell. Upcoming applications of MultiFate, when combine with other synthetic circuits, could provide stem cells with a variety of fate choices during multicellular system development. Furthermore, the field is evolving beyond just creating novel tools of synthetic biology. Integrating various tools to regulate various developing mechanisms, i.e., pattern formation and cell sorting, will enable the creation of synthetic embryos and complex organoids *in vitro*. Additionally, the expanding toolkit will allow researchers to investigate the interaction between progressive mechanisms in multicellular structures. For instance, optogenetic tools for manipulating shape of tissues could shed light on how changes in shape effect other developmental aspects.

The primary objective of regenerative medicine is to reinstate the functionality of damaged tissues within the body. Recent approaches typically involve engineering cell outside the body and then reinserting them, including CAR-T therapy. While such methods have led to significant improvements in patient outcomes, the need for meticulous *in vitro* processes introduces potential safety issue, like immunogenicity, and raises the cost of treatments. An alternative is the genetic

circuit delivery to stem cells *in vivo*, which could address such problems. Successful *in vivo* delivery requires vectors that are efficient, specifically targeted, and exhibit minimal immunogenicity. Current gene delivery systems include viral vectors, such as adeno-associated viruses (AAVs) and their derivatives, as well as non-viral vectors like lipid nanoparticles (LNPs), extracellular vesicles (EVs), and virus-like particles (VLPs) [70–72]. While these technologies have shown promise in both pre-clinical and clinical settings, further development of gene delivery technologies is necessary to enhance the *in situ* regeneration of damaged or multifunctioning tissues.

CONCLUSION

Pluripotent stem cell (PSC) therapies show great potential for driving advancements in regenerative medicine, largely due to their ability to self-renew and differentiate into various cell types. However, despite considerable progress, key obstacles, such as immune rejection, tumor formation, and achievable reliable cell differentiation, still hinder widespread clinical application. Synthetic biology offers innovative solutions through tools like synthetic receptors and genetic circuits, enabling precise control of cellular behavior, differentiation, and the development of multicellular systems. Progress in engineering cell lines, improving gene-editing techniques, and enhancing organoid and tissue production will be essential for addressing these limitations. Moving forward, research should prioritize optimizing *in vivo* delivery mechanisms and integrating synthetic biology with stem cell engineering to develop safer, more effective cell therapies and tissue regeneration strategies. Cross-disciplinary collaboration will be vital to fully harness the therapeutic potential of PSCs.

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