Advanced synthetic biology tools for genetic modification of human stem cells and their applications

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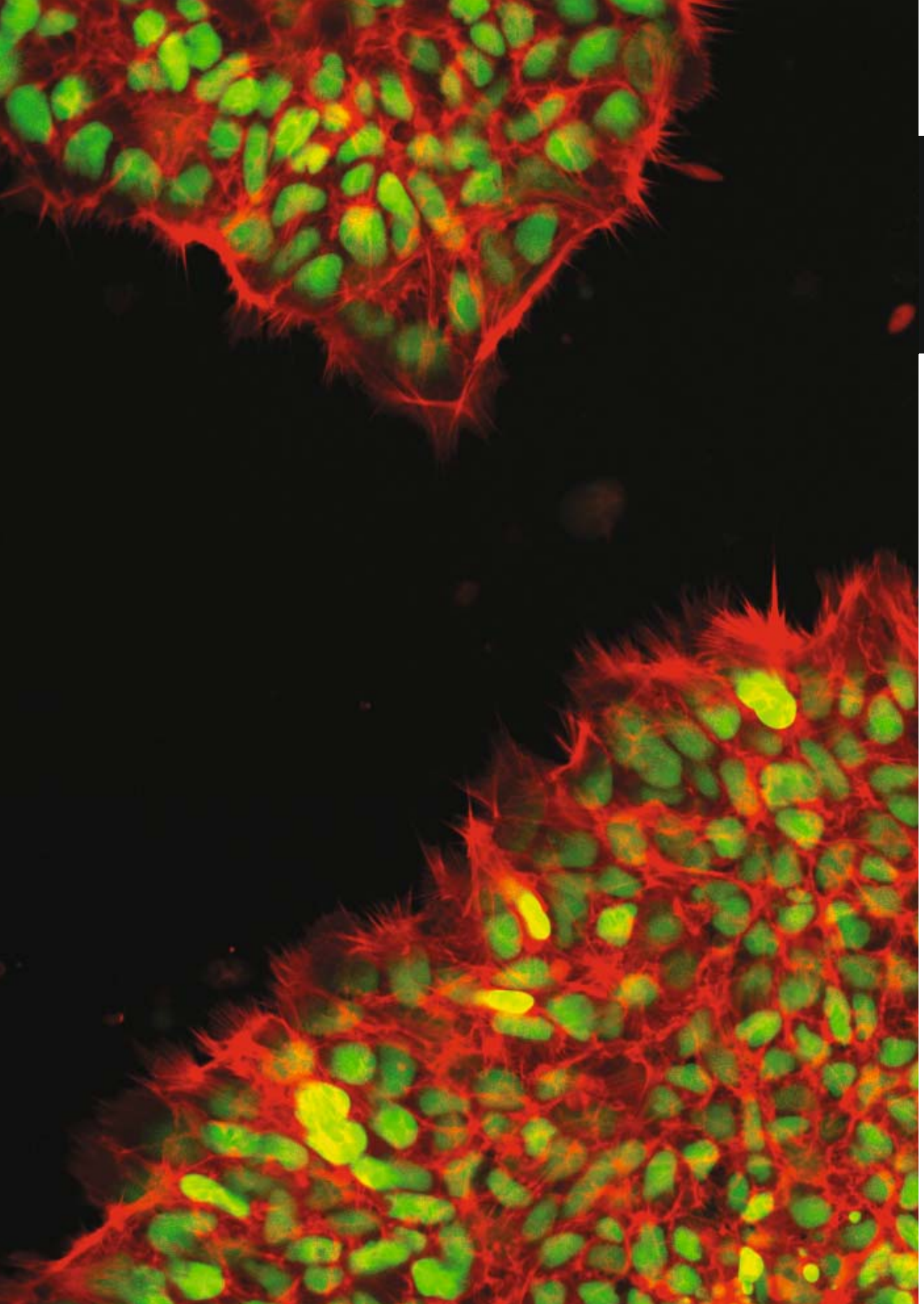

CHAPTER 9

General discussion and future perspectives

Introduction

The integration of large genetic payloads, particularly those exceeding 10 kb, into mammalian cells, including human induced pluripotent stem cells (hiPSCs), remains complex and the choice of integration approach is critical to success. Typically, a balance must be struck between integration efficiency and predictability. Random integration techniques offer high efficiency and rapid processing times but come with considerable unpredictability regarding copy number and the site of the integration. In contrast, targeted integration approaches, offer precise single-copy control and homogeneity within the cell population but involve more tedious procedures such as single-cell isolation and they achieve only moderate efficiency. One way to approach this is to install a landing pad (LP) in the cell line of interest and use this as a site to introduce genes of interest. This was the goal of this thesis which resulted in STRAIGHT-IN as a "go to" method for a variety of applications of hiPSCs. We believe that this platform has significant potential to drive progress in mammalian synthetic biology, improve biomanufacturing processes, and optimize cell therapy applications.

The genomic region where the LP is inserted plays a crucial role in determining the expression levels of subsequently integrated payloads. In human cells, genomic safe harbor (GSH) loci are frequently used, with *AAVS1* being a popular choice due to its well-characterized integration stability (Duportet et al., 2014; J. Li et al., 2020; Rosenstein et al., 2024). However, the main limitation with this locus in hiPSCs is the transgene silencing that occurs during differentiation into other cell types (Bhagwan et al., 2019; Blanch-Asensio et al., 2022; Ordovás et al., 2015). In **chapter 4**, in our initial studies involving the installation of landing pads (LPs) in hiPSCs, we utilized the *AAVS1* locus. However, we ultimately transitioned to the *CLYBL* locus, as it demonstrated higher expression levels of the same reporter in direct comparison with *AAVS1* (Blanch-Asensio et al., 2023; Cerbini et al., 2015). Although fluorescent reporters in the *CLYBL* locus generally outperformed those at *AAVS1* in terms of transgene expression levels, a subtle degree of silencing or reduced functionality of genetic circuits in differentiated cells was still observed (Blanch-Asensio et al., 2024). Targeting alternative GSH loci, such as *Rogi1* or *Rogi2*, could address these challenges (Aznauryan et al., 2022), although their efficacy in supporting robust transgene expression across different differentiated cell types remains to be determined. A recent study

computationally identified 25 potential GSH loci and experimentally validated three of them—Pansio-1, Olonne-18, and Keppel-19—by installing a LP into these loci in human embryonic stem cells (hESCs). Following integration of the fluorescent reporter Clover, all three loci supported robust transgene expression in both hESCs and differentiated cardiomyocytes, with Keppel-19 showing higher expression levels when compared to the other two loci (Autio et al., 2024). It is important to note that this evaluation was conducted using fluorescent reporters. It would be valuable to investigate whether this approach can be extended to other transgenes and proteins with greater therapeutic potential. Alternatively, installing LPs within housekeeping genes, such as *GAPDH*, is another intriguing possibility (Kao et al., 2016). This study employed a gene trap strategy where the gene of interest was tagged to the end of *GAPDH*. By carefully designing the insertion site, it might also be possible to insert a LP after the housekeeping gene, ensuring consistent expression across all cell types. This approach would leverage the stable and ubiquitous expression profile of *GAPDH* while introducing flexibility for integrating specific genes of interest. Nevertheless, this approach carries a risk that the integrated payload may interfere with normal gene function or disrupt expression levels within essential housekeeping genes.

As described in **chapter 4**, another potential strategy involves targeting genes that are specifically expressed in certain progenitor or differentiated cell types; for instance, we replaced an entire copy of *KCNH2* with a LP. We subsequently integrated this gene as wild-type or patient-specific disease variants of the gene, relevant for modeling long QT syndrome (Blanch-Asensio et al., 2022). This allowed us to simultaneously generate >10 disease hiPSC lines in a single well. A similar strategy could be performed in which LPs are inserted to the end of the regulatory elements of cell-type specific genes, without replacing the gene.

The process of installing LPs in the genome is the most labor-intensive aspect of platforms using this approach. This region will serve as the basis for subsequent DNA payload integrations. Thus, it is essential that these regions support high basal expression levels. We utilized CRISPR/Cas9 to insert LPs into the *AAVS1* locus and TALENs for the *CLYBL* locus, though the efficiency remained modest, with only ~1% of cells having dim reporter expression two passages after DNA delivery. Developing a set of hiPSC lines, each with a single copy of a standard

LP design integrated at distinct genomic sites, would facilitate a systematic comparison of these loci. In addition, such a panel would enable identification of optimal integration sites for specific differentiated cell types, including cardiomyocytes and neurons.

Nonetheless, the efficiency of conventional knock-ins in hiPSCs requires improvement to make it more feasible to establish panels of lines. Activation of p53 induces cell cycle arrest, facilitating DNA repair or triggering apoptosis to prevent the propagation of cells with severe DNA damage such as the cells with double-strand breaks (DSBs). Incorporating a dominant-negative fragment of p53 (p53DD) has been shown to increase the incidence of homologous recombination, facilitating the insertion of the DNA template at the DSB induced by the Cas9 endonuclease (Haapaniemi et al., 2018; Park et al., 2024). Remarkably, using iCas9 modRNA—a modified RNA construct that combines Cas9 and p53DD—yields higher editing efficiencies in hPSCs (Haideri et al., 2024). In fact, in **chapter 7**, we demonstrated that the addition of p53DD can also increase the number of cells that integrated the DNA payload via Bxb1-mediated recombination (Blanch-Asensio et al., 2024). However, the edited cells must be thoroughly characterized to ensure the absence of additional genomic abnormalities. Using a donor template incorporating a double ΔTK negative selection system can also further enhance on-target HDR-mediated knock-ins. The addition of GCV eliminates cells with undesired events, such as incomplete recombination (Nakade et al., 2023).

The expression of site-specific recombinases (SSRs), such as Cre recombinase, in human cells poses risks associated with unintended genomic alterations. These enzymes, while powerful tools for targeted genetic modifications, can induce off-target effects, including unintended chromosomal rearrangements, deletions, and translocations. Cre recombinase, in particular, is known for its potential to mediate recombination events at cryptic or pseudo-recognition sites, leading to genomic instability (Schmidt et al., 2000). To address this, the development of novel versions of the STRAIGHT-IN platform included transitioning from Cre-expressing plasmid to Cre modRNA and TAT-Cre protein. These alternative delivery methods significantly reduce the duration of Cre activity and presence within the cells compared to circular plasmids, which persist longer and maintain ongoing expression. This transient expression minimizes the potential for off-target effects, making modRNA and protein delivery safer

options. Additionally, Bxb1 was selected as the site-specific recombinase (SSR) for DNA payload integration, partly because of the absence of attP and attB pseudo-sites in the human genome, further enhancing safety. However, in some experiments, Cre and Flp recombinases were utilized to mediate the excision of auxiliary elements. To thoroughly evaluate the off-target effects of these SSRs, advanced genomic tools are essential. For example, Rec-seq, a technique that rapidly and unbiasedly profiles SSR DNA specificity using in vitro selection and high-throughput DNA sequencing, offers valuable insights into the safety and precision of recombinase activity for both therapeutic and research applications (Bessen et al., 2019).

DNA delivery into mammalian cells, especially for large constructs such as circular DNA plasmids, remains challenging. Transfection of plasmids larger than 10-15 kb is particularly problematic, often showing markedly lower efficiencies, which limits their practical use for complex genetic modifications with multiple genetic components. This inefficiency becomes even more pronounced with larger constructs, such as bacterial artificial chromosomes (BACs) that can exceed 100 kb in size. Current delivery methods, including lipofection and electroporation, show limited success with these larger constructs, and often cause cell toxicity, reduced viability, or low integration rates. In **chapter 4**, we successfully integrated multiple donor plasmids exceeding 50 kb in size, including a BAC construct of over 170 kb. However, the recovery of colonies following positive selection was significantly lower compared to smaller plasmids, which may impose limitations on performing multiplexed transfections with a larger number of >30-50 kb donor constructs. Alternative methods, like viral vectors, have size constraints on the payload they can carry that preclude their use with such large DNA sequences (Bulcha et al., 2021). Microinjection, although precise, is labor-intensive and not scalable (Low et al., 2022). There is a pressing need for innovative delivery systems capable of accommodating large DNA payloads with high efficiency and low cytotoxicity to facilitate advancements in genetic engineering. Emerging technologies, such as nanoparticle-based delivery and synthetic carriers (Duan et al., 2021; Zhu et al., 2022), hold promise but require further optimization to become viable for routine use with large DNA constructs. Advancing these DNA delivery methods is essential for expanding genetic capabilities in mammalian cells and enabling more complex, precise genetic modifications that are necessary for fields such as regenerative medicine, cell therapy and biotechnology.

Applications

We have demonstrated the technology developed in this thesis to be versatile, supporting a broad spectrum of applications, including drug screening and disease modeling (**chapter 4**), forward programming (**chapter 7**), and lineage tracing (**chapter 8**). While many of the examples reported here were primarily proof-of-principle experiments, these initial findings underscored the significant potential of the STRAIGHT-IN platform in a multiplicity of applications, with several of these being further investigated and the platform also utilized in other projects to address specific research questions (Meraviglia et al., 2024).

In **chapter 3**, we synthesized modRNA from genetically encoded voltage and calcium indicators. These modRNAs were efficiently transfected into a monolayer of hiPSC-derived cardiomyocytes, enabling repeated measurements of action potentials and intracellular calcium transients for up to seven days post-transfection (Yiangou et al., 2022). This approach facilitated both acute and long-term assessments of several compounds associated with cardiovascular risk. However, extending the monitoring period beyond seven days was not feasible without a second round of modRNA transfection. Additionally, variability in expression levels was observed, including a progressive decline in signal over time and we could not reach ~100% transfection efficiencies, therefore, some of the cardiomyocytes were not expressing the reporters. To address this, in **chapter 4**, we developed a multi-parameter reporter hiPSC line capable of simultaneously recording action potentials, intracellular calcium transients and contraction upon differentiation into hiPSC-derived cardiomyocytes (Blanch-Asensio et al., 2022). Drug-induced cardiotoxicity accounts for approximately 28-45% of all drug withdrawals from the market, primarily due to adverse effects on cardiac function such as arrhythmias or myocardial damage (Feng Sun and Shusen Sun, 2023). The genetically modified hiPSC line generated in this study, with its multi-parameter recording capability, provides a powerful tool for drug screening of both acute and long-term exposure of compounds. It complements earlier platforms in which these parameters were measured simultaneously using calcium- and voltage sensitive dyes and a membrane label, or by using modRNA (Meer et al., 2019; Yiangou et al., 2022). This genetic platform also enables detailed evaluation of cardiotoxicity, facilitating the identification of compounds with adverse effects on the heart and supporting safer drug development.

hiPSCs have revolutionized disease modeling since they can carry disease-causing mutations (either from a patient or introduced by genetic engineering) and can differentiate into any cell type of the human body. Even though these differentiated cell types are generally immature in phenotype, they can often capture mutation-induced disease features observed in patients (Bellin et al., 2013; Brandão et al., 2020). However, their practicality is limited when many mutations need to be introduced into hiPSCs or when multiple somatic cells carrying mutations require reprogramming. This is essentially what pharmaceutical companies would like to implement for drug screening. Not many drug response data points from derivatives of a single (diseased)-hiPSC line, but the responses of a few drugs in many hiPSC lines with genetic diversity: essentially, a clinical trial in a dish (Wu et al., 2024). In this thesis, we applied the STRAIGHT-IN platform to integrate full-length copies (>50 kb) of the *KCNH2* gene containing specific disease-associated variants, successfully generating 11 distinct hiPSC disease lines within a two-month timeframe (Blanch-Asensio et al., 2022). These included variants linked to either long QT or short QT syndromes, as well as variants of uncertain significance (VUS). Several of the variants that were followed up and analyzed were found to faithfully recapitulate the disease phenotype observed in patients carrying these same mutations. Importantly, all hiPSC lines described here were established in an identical genetic background, enabling systematic and direct comparison across variants. This approach is being extended to other genes, such as *LMNA* (Meraviglia et al., 2024), and we anticipate it can be broadly applied to other studies, particularly those requiring the introduction of multiple variants (>5) within the same gene.

An alternative approach involves knocking out one or both copies of a specific endogenous gene and reintroducing its coding sequence, either as a wild-type or mutated variant. This can be achieved through constitutive expression using conventional promoter sequences or controlled systems, such as the doxycycline-inducible Tet-On system. This would enable to investigate how variations in gene dosage can influence various biological processes. For example, it could provide insights into the role of these variations in driving direct cellular differentiation or contributing to the underlying mechanisms of disease.

These inducible systems can also facilitate the overexpression of transcription factors which can serve as master regulators of cell fate. This approach is known as forward or direct programming. Examples include *NGN2* for neuronal differentiation, *MYOD1* for skeletal myocyte development, and *ETV2* for endothelial cell specification (Pawlowski et al., 2017; Rieck et al., 2024). This strategy can markedly accelerate differentiation protocols, reducing the required time for differentiation by several fold and enabling the derivation of pure cell populations that are typically unattainable through conventional differentiation protocols (Pawlowski et al., 2017; Speicher et al., 2022). This approach commonly uses the Tet-On system to control expression of the transcription factor(s). In **chapter 7**, we initially assessed the influence of gene syntax—the order and orientation of genes—on the performance of the inducible system, allowing us to identify optimal configurations. Using one of the optimal designs, we overexpressed *NGN2* in hiPSCs to drive rapid differentiation into neurons (Blanch-Asensio et al., 2024). We foresee that this platform could support more advanced forward programming strategies to generate rare cell populations or novel cell types that have previously been inaccessible. Among these are motor neurons, sympathetic and parasympathetic neurons and pacemaker cells. However, identifying suitable transcription factors is a prerequisite for this. Various platforms leveraging transcriptomic data can be utilized for preliminary screening to identify potential candidates (Jung et al., 2021; Rackham et al., 2016).

Finally, in **chapter 8** we utilized the Tet-On system to control the expression of programmable endonucleases including Cas9. While still in preliminary stages, our findings demonstrate the potential of this approach to facilitate lineage tracing applications through scar formation as previously demonstrated in cancer cells and *in vivo* (Bowling et al., 2020; McKenna et al., 2016; Yang et al., 2022). When integrated into monolayer or 3D differentiation protocols that yield multiple cell lineages, this method can provide valuable insights into the developmental origins of various differentiated cell types, enabling identification of lineage relationships and improving our understanding of cellular differentiation pathways.

Among the applications highlighted, we believe that our newest variant on this technology, STRAIGHT-IN Dual (**chapter 7**), has the capacity for controlled, multi-gene integrations within a stable genetic background, positioning it as

a tool for a range of biomedical fields where reproducibility, precision, and scalability are critical. For instance, addressing research questions such as i) How do different combinations of genes or regulatory elements interact within the same cell to influence gene expression or cellular behavior? ii) How can complex gene circuits be optimized in a high-throughput manner in hiPSCs or derived cell types? And iii) How can targeted, multi-loci integrations influence cellular differentiation pathways and lineage specification?

To address this, it is necessary to enhance the high-throughput capacity for generating a large number of genetically modified lines in a single transfection, as previously demonstrated in **chapter 4** with the multiplexed integration of 12 KCNH2 plasmids (Blanch-Asensio et al., 2022). These could be answered by using a similar approach to CLASSIC, involving the use of extensive plasmid libraries comprising thousands of diverse components that are integrated at a single-copy into *AAVS1* and the sequence-to-function relationship is mapped via long- and short-read next-generation sequencing (O'Connell et al., 2023). While this platform has been optimized to identify optimal genetic designs for transgene expression, it could be further adapted and expanded using STRAIGHT-IN or more advanced versions of the platform, which would enable precise and efficient genetic modifications tailored to these applications. To fully realize this, however, it is essential to first expand the currently limited molecular toolbox available for hiPSCs and hiPSC-derived cell types, which could also be addressed using large plasmid libraries containing these components. Additional details regarding these advancements and demands are outlined in the following section, *Future Perspectives*.

Future perspectives

Additional STRAIGHT-IN hiPSC lines

Establishing a hiPSC line with two orthogonal LPs controlled by a single serine recombinase (Bxb1) for dual DNA payload integration and a single tyrosine recombinase (Flp) for excision of auxiliary elements from both LPs would represent a significant advancement. Our current STRAIGHT-IN Dual line uses a single a serine recombinase but requires two tyrosine recombinases, Cre and Flp. To address this, the existing GT-allele would require redesigning to include two additional heterospecific *FRT* sites. Currently, the GA allele contains *FRT* and *F3* sites; however, *F13* and *F14* sites have been shown to maintain

orthogonality with these existing sites with similar efficiencies, making them a logical choice for testing (Turan et al., 2010).

In recent years, several novel SSRs have been identified or engineered, demonstrating improved performance compared to existing recombinases (Durrant et al., 2023; Jelicic et al., 2023). Additionally, mutations have been introduced into recombination sites to identify orthogonal counterparts that are compatible with different recombinases, thereby enabling multiplexed applications. These modifications not only facilitate the pairing of recombinases with specific, non-interfering recombination sites but also serve to enhance recombination efficiency, optimizing the precision and effectiveness of site-specific integration (Cautereels et al., 2024; Jusiak et al., 2019). Given the labor-intensive nature of LP installation, the STRAIGHT-IN platform offers a significant advantage by allowing LP replacement with counterparts that incorporate recombination sites specific to these new SSRs. For example, this novel LP may carry other mutant *Bxb1* attP sequences or recombination sites for other integrases, and could be inserted as the DNA payload. Upon successful excision, the original LP is replaced with a completely new LP, retaining only the sites required for the excision of auxiliary elements. This approach would facilitate systematic comparisons of different SSRs within an identical genomic context, enabling precise evaluation of their relative efficiency and functionality. Through this method, highly efficient SSRs can be identified and optimized, providing candidates for further rounds of directed evolution aimed at enhancing integration efficiency. This strategy has shown recent success, exemplified by improved integration efficiencies achieved with an evolved version of Bxb1 recombinase (Hew et al., 2024; Pandey et al., 2024). The use of such advanced SSRs holds promise for refining genome engineering techniques, increasing both precision and efficacy in complex genetic modifications.

An intriguing alternative approach would involve installing LPs into adult stem cells (ASCs), such as hematopoietic stem cells or intestinal stem cells. Although ASCs exhibit more limited differentiation potential compared to the pluripotency of hiPSCs, they offer several distinct advantages. For instance, the resulting derived cells are more mature with tissue specificity, well-established and validated culture conditions and shorter timelines for clinical translation (e.g. bone marrow transplants) both for regenerative medicine (Gurusamy et al., 2018; J. Li et al., 2022) and (genetic) disease modeling (Geurts et al., 2023; Geurts &

Clevers, 2023). This established role in regenerative medicine positions ASCs as promising candidates for LP installation, potentially streamlining the path from research to clinical application although it will be challenging to ensure that the population with extended lifespan are actually targeted.

Expanding the molecular toolbox for hiPSCs

The latest version of the STRAIGHT-IN platform, described in **chapter 7**, offers an approach for DNA integration, providing single-copy control of two DNA payloads with high efficiency in a short timeframe. After optimizing the protocol, we achieved approximately a 10-fold increase in integration efficiency prior to enrichment, reaching the stage that nearly 100% of cells carry a single-copy of the payloads in each allele of *CLYBL* following antibiotic selection. For the GA allele, we also observed ~100% excision efficiency for all the auxiliary elements, completing the entire protocol in a single cell culture well and in under 10 days (Blanch-Asensio et al., 2024).

Given the limited molecular toolbox (i.e. genetic components designed to manipulate the genome with high precision and efficiency) available for hiPSCs, STRAIGHT-IN presents an excellent platform for conducting high-throughput screening of large plasmid libraries, enabling the identification of novel DNA elements optimized for use in hiPSCs and derived cell types (**Figure. 1**). Similar studies have been conducted in HEK293T cells, although they primarily focused on refining established genetic components to determine optimal designs for transgene expression (O'Connell et al., 2023). Although a more extensive list of components could be identified, the genome engineering of hPSCs could be significantly enhanced through the expansion of promoter sequences, insulator elements and inducible systems.

Promoter sequences

Identifying promoter sequences for sustained transgene expression in hiPSCs remains a significant need. In this study, we used the pGK promoter to drive expression of fluorescent markers from the LP; however, expression levels declined to undetectable after a few passages of the isolated clones. Similarly, in **chapter 7** we observed that the hEF1a promoter was prone to silencing, with only a few cells maintaining transgene expression after several passages (Blanch-Asensio et al., 2024). Other commonly used promoters, such as ACTB,

CMV, SV40, UbC, and EFS, either exhibit weak expression or also undergo silencing over time in hPSCs (Eggenschwiler et al., 2013; Norrman et al., 2010). Currently, only the CAG promoter has demonstrated strong expression in hiPSCs and derivative cell types (Blanch-Asensio et al., 2022, 2023, 2024; Karbassi et al., 2024; Pawlowski et al., 2017). It remains to be seen whether this gap can be addressed using artificial intelligence for the identification of novel promoter sequences. There is a clear need to identify a variety of promoters that are stable while enabling differential expression levels (i.e., low, medium, and high) in hiPSCs, and are resistant to silencing across most differentiated cell types. For this, insulator elements can be placed upstream of the promoter sequence to mitigate silencing (Müller-Kuller et al., 2015) although it remains unclear whether this can be translated to every cell type of the human body.

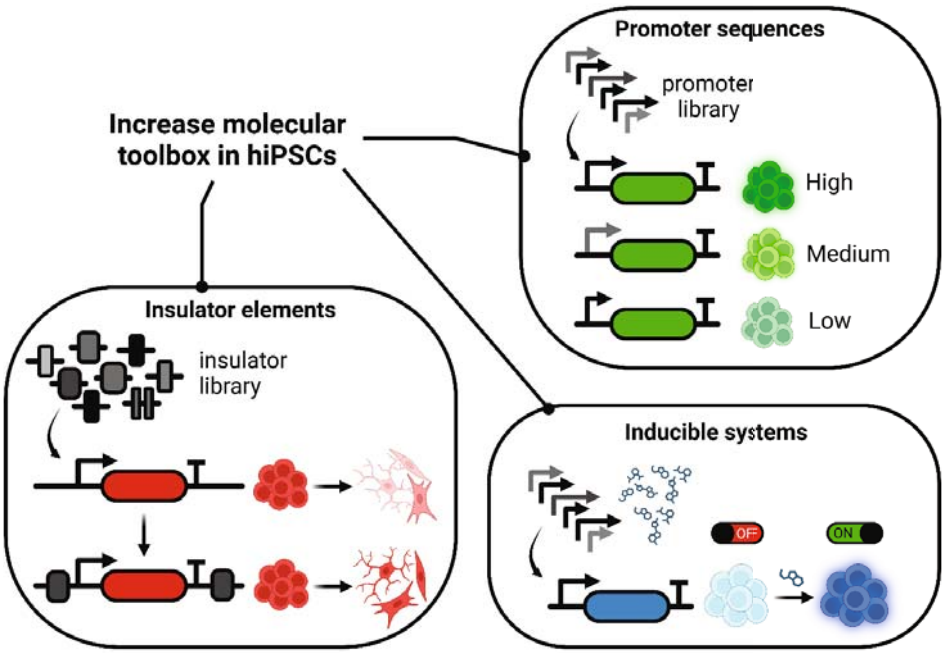

Figure 1. Challenges and limitations of key molecular components for genome engineering in hiPSCs.

This figure highlights the challenges and limited availability of three essential molecular components for effective genome engineering in hiPSCs: promoter sequences, insulator elements and inducible systems. Promoters are scarce and prone to silencing and insulator elements require further validation to prevent transgene silencing during differentiation to multiple cell types. Inducible systems offer controlled gene expression, yet often face issues like leakiness and toxicity of the inducer. Collectively, these challenges emphasize the need for more robust, reliable molecular tools specifically adapted to hiPSCs to support stable and versatile transgene expression for diverse applications.

Insulator elements

Transgene silencing is a persistent issue during the differentiation of hiPSCs into various cell types. Transgenes integrated into GSH regions often exhibit high expression in undifferentiated cells, but their expression can diminish significantly or even be fully suppressed in differentiated cells. Another approach to counteract transgene silencing involves the use of chromatin insulator sequences that function as genomic barriers. While these elements are thought to mitigate silencing (Müller-Kuller et al., 2015; Zhang et al., 2023), there is currently no consensus on their effectiveness or on which specific sequences they are most suitable. Recently, Zhang et al. conducted a high-throughput screen of a thousand potential barrier elements, identifying eight candidates with insulator properties comparable to the commonly used cHS4 sequence (Zhang et al., 2023). However, this study was conducted in HCT116 cells, which do not have the ability to differentiate, and involved a relatively small number of elements. This highlights the need for similar screening studies in hiPSCs, followed by differentiation into cell types from the three germ layers, to comprehensively assess transgene silencing and identify effective insulator elements.

Inducible systems

The vast majority of transgene overexpression systems that are inducible in mammalian cells rely on the Tet-On mechanism, using tetracycline or doxycycline as inducer. Recently, alternative systems such as NS3, ERT2, and ABI-PYL, which are inducible by FDA-approved small molecules like grazoprevir (GZV), 4-hydroxytamoxifen (4-OHT), and abscisic acid (ABA), respectively, have been evaluated in human cell lines and primary T cells (H.-S. Li et al., 2022). In this thesis, we assessed the GZV-inducible NS3 system in hiPSCs, observing no leakiness and good induction levels. However, GZV showed some toxicity in undifferentiated cells (Blanch-Asensio et al., 2024). The ERT2/4-OHT system is known to have potential leakiness due to its mechanism based on nuclear translocation (Korecki et al., 2019; H.-S. Li et al., 2022). Additionally, the ABI-PYL/ABA system exhibited only a modest increase (fold-change) in expression upon induction (H.-S. Li et al., 2022). An alternative system, X^{on}, which operates through drug-induced splicing, offers promising potential with LMI070 as an inducer (Monteys et al., 2021). Recently, a miniaturized version of this drug-induced splicing switch, termed MiniX^{on}2G, was successfully established in

hiPSCs, expanding the toolkit of inducible systems in these cells (Chi et al., 2024).

Spatiotemporal control, such as the use of light as an inducer, presents an intriguing alternative approach. For example, light-activated Spyligation allows for the irreversible and stable reassembly of two non-functional split fragments into a functional protein (Ruskowitz et al., 2023). This method has been successfully applied to fluorescent proteins, luciferases, and even Cre recombinase. However, its broader applicability to all proteins of interest appears to be limited, as it requires splitting the target protein into fragments that regain functionality only upon reassembly. Despite these advances, there remains a need for inducible systems with the following properties: (i) no cell toxicity induced by the small molecule, (ii) no leakiness in the absence of the inducer, (iii) a large "fold-change" between on and off states, and (iv) rapid kinetics upon addition and removal of the inducer.

Conclusion

In conclusion, integrating large genetic constructs into hiPSCs remains challenging, requiring a careful balance of efficiency, precision, and stability. While rapid integration methods often lack control, targeted approaches provide single-copy accuracy but with reduced efficiency. In this thesis, a novel DNA integration platform is described that shows a promising balance among these constraints, though there is still room for improvement, particularly in optimizing steps and exploring additional GSH regions for LP installation. Overall, we anticipate this method will serve as a robust platform for reliable hiPSC genetic engineering, supporting diverse research and therapeutic applications while expanding the molecular toolbox available for hiPSCs.

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