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The past, present, and future promise of pluripotent stem cells

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1. Background and promise of pluripotent stem cells

James "Jamie" Thomson's isolation of non-human primate[1] and human embryonic stem cells (ESCs)[2] in the mid-to-late 1990s ushered in a new era for regenerative medicine. ESCs–the first type of human pluripotent stem cell (PSC) to be isolated and sustained in cell culture–can be scaled-up and differentiated *in vitro* into vast quantities of cells representing any tissue in the human body, with the exception of extraembryonic tissues (e.g., placenta). As a result of Thomson's achievement, a tremendous amount of attention[3] and resources began to flow into ESC research in the 2000s. The ultimate goal, and *raison d'etre* for many careers, became clear: use ESCs to generate curative cell therapies for a number of diseases.

Building upon existing developmental biology literature, scientists went on to develop many protocols for generating cell types from ESCs representing all three germ layers, including cardiomyocytes,[4] neurons,[5] hepatocytes,[6] endothelial cells,[7] thymic epithelial cells,[8] and hematopoietic cell types.[9] Of particular promise was the idea of making entire PSC-derived organs to supplant the need for cadaveric and living-donor solid organ transplants. Additionally, researchers and patients envisioned the revolutionary potential of PSC-derived hematopoietic stem cells (HSCs) to overcome key barriers in bone marrow transplantation, including cell scarcity, donor availability, and age-related factors.[10] The possibility of eliminating transplant wait-lists, expanding therapies for cancer patients, and creating cures for devastating spinal cord injuries, for example, were at the forefront of many minds.

In addition to optimizing differentiation protocols for making new PSC-based cell therapies, multiple groups utilized ESCs as an investigative tool to better understand human development and the biological underpinnings of pluripotency. Building upon this knowledge, in 2006 Shinya Yamanaka's group identified key transcription factors driving pluripotency and forced their overexpression in mouse fibroblasts to create the first induced PSC (iPSC) lines.[11] This discovery merged the scalability and differentiation potential of ESCs with the advantages of an autologous (i.e., "self") genotype; it was now possible to generate individualized PSC-based cell therapies. Jamie Thomson's group at the University of Wisconsin-Madison was simultaneously exploring this avenue of research, bypassing mouse studies in order to focus on more the more clinically-relevant reprogramming of human cells. In 2007, Thomson[12] and Yamanaka[13] contemporaneously published the first human iPSC reports, with both groups using separate and overlapping combinations of pluripotency factors to achieve similar end results. The scientific community now had the foundational building blocks to make human patient-specific cell therapies for a number of diseases and, critically, these cells might avoid the allorejection that has been so detrimental to traditional solid organ and HSC transplant patients. The emerging potential and promise of a new era of medicine captured the minds of researchers, funding agencies, industry, and countless patients worldwide.

At the time of this writing, the regenerative medicine field has just marked the 25-year anniversary of Thomson's seminal publication of human ESCs. We unabashedly take the view in support of the continued promise of PSC research and the potential for curative PSC-derived treatments to be realized within our lifetimes. Nonetheless, good science requires periodic comparison of the accumulated evidence versus original expectations in order to best determine future research strategies. Science is not immune to wishful thinking, hype, and bias of entrenched interests. Here, we therefore will address a prominent critique of the field: the failure to-date to transplant lab-grown organs or to conclusively demonstrate curative PSC-derived therapies in the clinic. We will also discuss the key biological concept of cellular maturation, which is relevant to the eventual widespread clinical application of PSC-therapies. We will then examine how an exciting development in the field of genomic engineering has opened up new avenues for discovery in PSC research. Finally, we will comment on possibilities for the future, informed by our experience of actively participating in this research for more than a decade.[14–19]

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2. Current research in the shadow of original expectations

> "If you can look into the seeds of time, and say which grain will grow and which will not, speak then unto me."
>
> –William Shakespeare (from *Macbeth*)

Scientific breakthroughs articulate previously ineffable questions. The brightest minds quickly provide hypothetical answers, with additional questions and answers trickling out into the public discourse in short order, resulting in the emergence of a new research paradigm. Much of this is set in motion before a single follow-on experiment has been conducted. This is a well-established path of progress and it has its own inertia. This path also has pitfalls, including being prone to unrealistic timeline predictions, especially early on.

In the case of PSCs, some predictions have borne out *more* quickly than originally anticipated. After isolating human ESCs, Thomson focused his efforts on making iPSCs and thought he would spend the rest of his career working towards that goal.[20] In actuality, it took less than a decade to generate viable iPSCs. (Thomson went on to find new areas for pioneering work in basic and translational research before retiring in 2022).[21–24]

Other predictions have met the cold reality of the regulatory burdens and political impediments of translating bench PSC research to the clinic. As of 2022, there have been 137 clinical trials involving iPSCs. Of the 81 ongoing observational and interventional clinical trials, 62 were non-therapeutic (e.g., studies that used iPSCs in interventional procedures such as disease modeling but did not administer cell therapies to patients) and 19 were therapeutic in nature (i.e., involve administering iPSC-cell therapies to patients)[25] This is clear progress but not as quick as many would have hoped. An early-2000s governmental imposition limiting the number of PSC lines eligible for federal funding was arguably an additional contributing factor to the slower than expected pace of American progress in the field.[26] But the complexity of human biology itself was, and remains, the largest force dictating the pace of PSC research progress worldwide.

There are a number of biological factors that have complicated PSC research efforts, including suitable animal models for transplantation studies,[17,27–29] karyotypic stability,[30] cell cycle synchronization,[31] and the differentiation kinetics associated with developmental clocks.[32] Many of these have been addressed in detail elsewhere.[33] One critical factor that was not fully apparent in 1998 but gradually became clear after two decades of research is cellular maturation.[34]

3. The impacts of immaturity

Maturity of cells does not result strictly from age, but also is dynamically established in response to genetic and environmental cues in order to achieve maximal adaptation and specialization for specific tasks.[34–36] As PSC research progressed in the 2000s and 2010s, it became apparent for a number of different PSC-derived cell types that, despite having key phenotypic and functional hallmarks of terminally-differentiated cells, they may not always be fully-mature.[37–39] For example, there are multiple protocols for deriving PSC-hematopoietic stem and progenitor cells (HSPCs) capable of producing various blood cell lineages.[9,40,41] However, these cells typically represent the primitive hematopoiesis of the embryonic yolk sac, not the more mature definitive hematopoiesis of the aorta-gonad-mesonephros region. Yolk sack HSCs are biased towards embryonic hemoglobin instead of fetal or adult[42] and PSC-HSPCs (containing a mixture of HSCs and other blood progenitors) fail to reproducibly engraft in immune-deficient animals in sufficient numbers.[43] Despite this yet-insurmountable limitation, there are various strategies to promote the maturation of various PSC-derived target cells in the hematopoietic and other lineages. Two promising new publications could represent a critical advance in identification and differentiation of *bone fide* HSCs from PSCs.[44,45] However, these and any future reports of engraftable PSC-HSPCs need to be reproduced by independent investigators prior to concluding that this important hurdle has finally been overcome.

A case study of PSC-cardiomyocytes (CMs). For the purpose of this work, we examine the specific case of PSC-CMs and discuss various parameters and scientific advancements that are aimed at improving their cellular maturation and engraftment integrity. A significant obstacle in the application of current protocols for therapeutic human PSC-CMs is the resulting CMs resemble embryonic or fetal cells, not unlike the PSC-HSPCs mentioned above. These immature CMs exhibit notable differences in structure and function compared to adult CMs: abnormal morphology, smaller size, limited proliferation potential, reduced contractility, disorganized myofilaments, automaticity, depolarization, and low action potential upstroke velocity.[34,36,46–48] This is despite having a number of gene-expression, protein, and physiological similarities to primary CMs, which still makes these protocols attractive for clinical applications given the tremendous need for cellular therapy in multiple cardiovascular pathologies.[49] However, these cells are imperfect and optimizing maturation could allow for improved long-term integration and function of PSC-CM therapies.[36]

To address these limitations, various approaches have been explored to enhance the maturation of PSC-CMs, including long-term cell culture and engineering three-dimensional (3D) culture environments. One promising strategy involves electrical stimulation, as electrical signaling plays a crucial role in embryonic development. Several studies have demonstrated that electrical field stimulation increases the expression of key cardiac genes, improves phenotypes, and enhances calcium handling.[50–53] Another approach involves utilizing a Wnt signaling protocol with the addition of Polyinosinic-polycytidylic acid during the reprogramming of skin fibroblasts to iPSC-CMs.[54,55] This epigenetic priming method, as demonstrated by Biermann et al.,[54] accelerates reprogramming and yields CMs with increased maturity and larger size. Additionally, modifying the composition of the culture media has shown promise in enhancing PSC-CM maturation.[56,57] Such modifications have resulted in higher oxidative metabolism, improved calcium handling, and enhanced contractility. Conventional two-dimensional culture conditions in a static dish lack the complexity of *in vivo* environments, such as cell-cell interactions and extracellular microenvironments. To overcome this limitation, 3D cultures of PSC-CMs in the form of engineered heart tissue (EHT) using hydrogels or matrices have been developed.[58] The 3D EHT approach mimics the *in vivo* environment more closely, allowing for cell-cell interactions and mechanical tension development. This has been shown to significantly enhance maturation by making the CMs more responsive to biochemical and physical stimuli.[59,60] EHTs have demonstrated similar electrophysiological traits as adult ventricular tissue and improved functionality. Lastly, multicellular PSC-cardiovascular grafts are a promising approach that has shown distinct improvements in maturation of CMs.[61]

The ultimate goal of these maturation strategies is to achieve functionally and structurally mature CM suitable for clinical transplantation.[62] Recent studies with cells of varying degrees of maturity have shown promising results in animal models, such as injecting PSC-CMs into rats with myocardial infarction, which improved myocardial function and reversed ventricular remodeling.[63] Notably, in 2022 Miyagawa et al.[64] conducted a clinical trial where allogeneic iPSC-CM patches were implanted in a 51-year-old male patient with severe heart failure due to ischemic cardiomyopathy. Within six months, significant improvement in clinical symptoms was observed. Additional translational experimentation in this area will likely improve the performance of PSC-CM grafts further. While these developments may have taken more time than originally anticipated, the identification and implementation of methods for maturation of PSC therapies hold great promise for the treatment of a wide range of cardiovascular and other diseases.

4. Synergistic science: gene-edited PSCs

In the last two decades, remarkable progress has been made developing tools to permanently modify the genetic code of mammalian cells. Zinc Finger Nucleases (ZFN) are targetable DNA cleavage reagents that induce double strand breaks, which are then taken over by the cellular DNA repair processes leading to targeted gene replacement. ZFNs have been implemented to correct site-specific defects in multiple types of stem cells. For example, ZFN-based site-correction in $CD34^+$ HSPC cells from patients with Sickle Cell Disease was shown to produce wild-type hemoglobin tetramers upon being grafted into NOD/SCID/IL2rγ null (i.e., NSG) immune-deficient mice, which were shown to further differentiate into myeloid, erythroid, and lymphoid cell types.[65] Transcription Activator-Like Effector Nucleases (TALEN)-based gene editing is an alternative to ZFN that uses artificial restriction enzymes to cut the DNA strand at specific sites by combining the DNA binding regions of transcription activator-like (TAL) effectors with DNA cleavage domains.[66] Multiple studies based on correction of monogenic disease defects have been reported using TALENs in PSC-derived cell types. For example, Sun et al.[67] reported TALEN-based correction of human hemoglobin beta (HBB) mutation cells from Sickle Cell Disease patient-derived iPSCs. In recent years, however, ZFN and TALEN gene editing in PSCs has been overtaken in popularity by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) editing.[68]

The CRISPR/CRISPR-associated protein 9 (Cas9) system cuts DNA at specific sites using guide-RNA with Cas9 enzyme, allowing gene knock-in or knock-out and natural DNA repair.[69] A study using CRISPR-mediated correction of HBB in iPSCs from patients with β-thalassemia showed that HBB expression could be restored in iPSC-erythroblasts.[70] Optimized CRISPR-based editing, such as the use of the CRISPR interference system with deactivated Cas9 nuclease, has also proven advantageous for gene editing applications. This system reduces transcriptional expression, thereby allowing for transcriptional regulation of the gene of interest without disrupting its function completely.[71] It was implemented in patient-derived iPSC-CMs that were generated with a mutation in CALM2, which codes for the $Ca2^+$ binding protein Calmodulin, a mutation that can lead to Long QT syndrome.[72] In this study, the group found that the CRISPRi-led reduction in CALM2 expression in wild-type and mutated CALM2 resulted in shorter action potential duration and faster $Ca2^+$/Calmodulin-dependent inactivation compared to untreated iPSC-CMs.

In addition to direct therapeutic applications, PSC gene editing has become a useful tool for studying the genetic defects that contribute to various diseases. Leveraging the pluripotent (i.e., being able to be differentiated into multiple cell types) nature of PSCs, researchers can perform edits in specific regions of a gene to study the resulting effects upon the PSCs and PSC-derived cells. For example, iPSC-derived hemophilia A disease models can be made by introducing a 140-kbp chromosomal segment inversion in the F8 gene.[73] When the segment was reverted to its original orientation, the wild-type F8 mRNA was detected from the gene-edited cells, suggesting a successful correction. In another study,[74] a KCNA5 knockout model created from iPSCs was used to demonstrate the role of Kv1.5 in determining electrophysiological properties of the iPSC-CMs. In addition to therapeutic gene correction and biological discovery, we and others see great promise in the use of gene-edited PSCs to create hypoimmune cell therapies that avoid recognition and elimination (i.e., allorejection) by the immune system.

5. Engineering tolerance or ignorance

The term hypoimmune gene editing refers to the application of gene editing technologies to alter genes or pathways involved in the immune response in PSC lines to achieve a state of immune system anergy, suppression, or other lack of effector response to PSC-derived grafts (i.e., immune tolerance). Importantly, this could include harnessing mechanisms of canonical self-tolerance or use of strategies to promote a more basic "ignorance" of transplanted grafts i.e., escape from effector immune recognition via a number of possible (disparate) mechanisms.[75] We[18] and others[76,77] have reviewed this topic in detail previously. As with solid organ transplantation, the goal of hypoimmune gene editing is to enable durable immune tolerance to a transplanted graft for the lifetime of a transplant recipient, ideally without the need for immunosuppressive drugs. The current gene targets for hypoimmune gene editing are the extracellular and intracellular molecules, and their products, that are used by the multiple cell types from the innate and adaptive immune system during the process of allograft rejection.

Gene editing techniques such as TALENs, ZFNs, and now CRISPR have been employed to target the HLA-I and –II molecules via multiple strategies, enabling the engineered cells to evade T-cell and donor-specific antibody responses. The highly polymorphic genes of HLA-I (HLA-A, -B, and –C) encode the alpha chains of the major histocompatibility molecules (MHC), which form on the surface of cells when complexed with β-2-microglobulin (B2M). Class I MHC presents peptide antigens to $CD8^+$ T cells and serves as targets for antibodies during allorejection. These processes can be rendered non-functional by targeting B2M for ablation, preventing any class I MHC from forming on the surface of cells (including those that inhibit natural killer cells, such as HLA-E). Multiple studies[78–81] have shown that the elimination of B2M in human ESCs effectively enabled evasion of cytotoxic $CD8^+$ T cell responses. In another study,[82] PSC-derived islet grafts devoid of B2M were protected for approximately two months when transplanted into immune-deficient mice. Similarly, HLA-II molecules (HLA-DP, -DQ, -DR) can be rendered non-functional by targeting the HLA-II Transactivator (CIITA),[83] which is a transcriptional regulator essential for HLA-II expression. HLA-II encodes the MHC that present antigens to $CD4^+$ T cells and are also targets for antibodies during allorejection. In another study,[84] it was shown that several differentiated cell types such CMs, smooth muscle cells, and endothelial cells generated from HLA-I and –II knock-out iPSCs survive within HLA-mismatched recipients in the absence of immune suppression. Additional gene-engineered modifications in conjunction with HLA knock-out PSC lines, such as over-expression of CD47 or an HLA-E fusion protein not dependent on B2M,[85] have been shown to prevent natural killer cell activity that will otherwise lyse cells due to the "missing-self" (i.e., absence of surface MHC). Importantly, hypoimmune PSC gene-editing strategies will need to balance prevention of rejection (by both innate and adaptive effector cells) with the need to protect the cells from becoming uncontrolled viral reservoirs and/or proto-tumors. Identification and validation of novel gene edits that are safe and effective at preventing allorejection is an active area of research for our group and others. In our view, careful study of over 50 years of transplantation immunology research literature along with interdisciplinary collaborations between PSC biologists, transplantation immunologists, and others will give the highest likelihood of success for developing clinically relevant hypoimmune PSC therapies. Undoubtedly, there are additional potentially promising approaches informed by collaborative experiences that could be interrogated in the coming years (e.g., exploring active tolerance induction towards iPSC-derived neoantigens).[86]

6. The future

We are currently living in a remarkable new phase of scientific discovery. As discussed above, a tremendous amount of progress has been made in the PSC field over the past 25 years and many of those advances have been bolstered by discoveries in other disciplines, such as with CRISPR/Cas9 gene editing. In addition to an improved understanding and manipulation of the mechanisms of pluripotency, as the PSC field matures further into the second half of this decade PSC researchers will no doubt benefit from new discoveries in other areas of research, such as artificial intelligence (AI). At the time of this writing, AI is being steadily integrated into academic and industrial biomedical research pipelines,[87]

and as a society we are witnessing a rapid "democratization" of this technology that will impact the training and capabilities of the next generation of scientists.[88] It is possible that by the end of this decade, AI's evolution will significantly impact how researchers conduct all aspects of science, including regenerative medicine.[89] We anticipate that AI could reasonably be used to: interpret large transcriptomics datasets to solve mysteries of human development; design counterintuitive experiments that overcome key barriers to cellular maturation; and determine unorthodox gene edits that enable immune-tolerated PSC therapies to persist long-term in transplant patients.

In addition to learning from other disciplines, multidisciplinary collaboration will be an important engine of progress for the PSC field. Translational research teams that include PSC biologists, transplantation immunologists, bioengineers, and clinicians will be well-poised to create HLA-matched lab-grown organs devoid of the passenger lymphocytes implicated in the direct pathway of allorecognition. Ischemia reperfusion injury, which has bedeviled traditional solid organ transplantation for over 50 years, may be minimized by optimizing PSC transplant design and timing for individual patients in light of their particular disease status and health status. GMP-grade manufacturing laboratories incorporated into hospitals, similar to those increasingly used for making point-of-care chimeric antigen receptor therapies,[90] could allow physicians to choose the optimal time for transplantation of patient-specific PSC grafts. These and other possibilities are now coming into focus, but it is still premature to speculate on definitive timelines for regulatory approval and widespread use of specific treatments. We do, however, anticipate that today's momentum of increasing numbers of investigational new drug applications and clinical trials will translate into a curative PSC therapy that is widely-available by the end of this decade for one or more diseases.

Declaration of competing interest

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Data availability

No data was used for the research described in the article.

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