Advancing cardiac safety and drug discovery screening using human stem cell-derived cardiomyocytes

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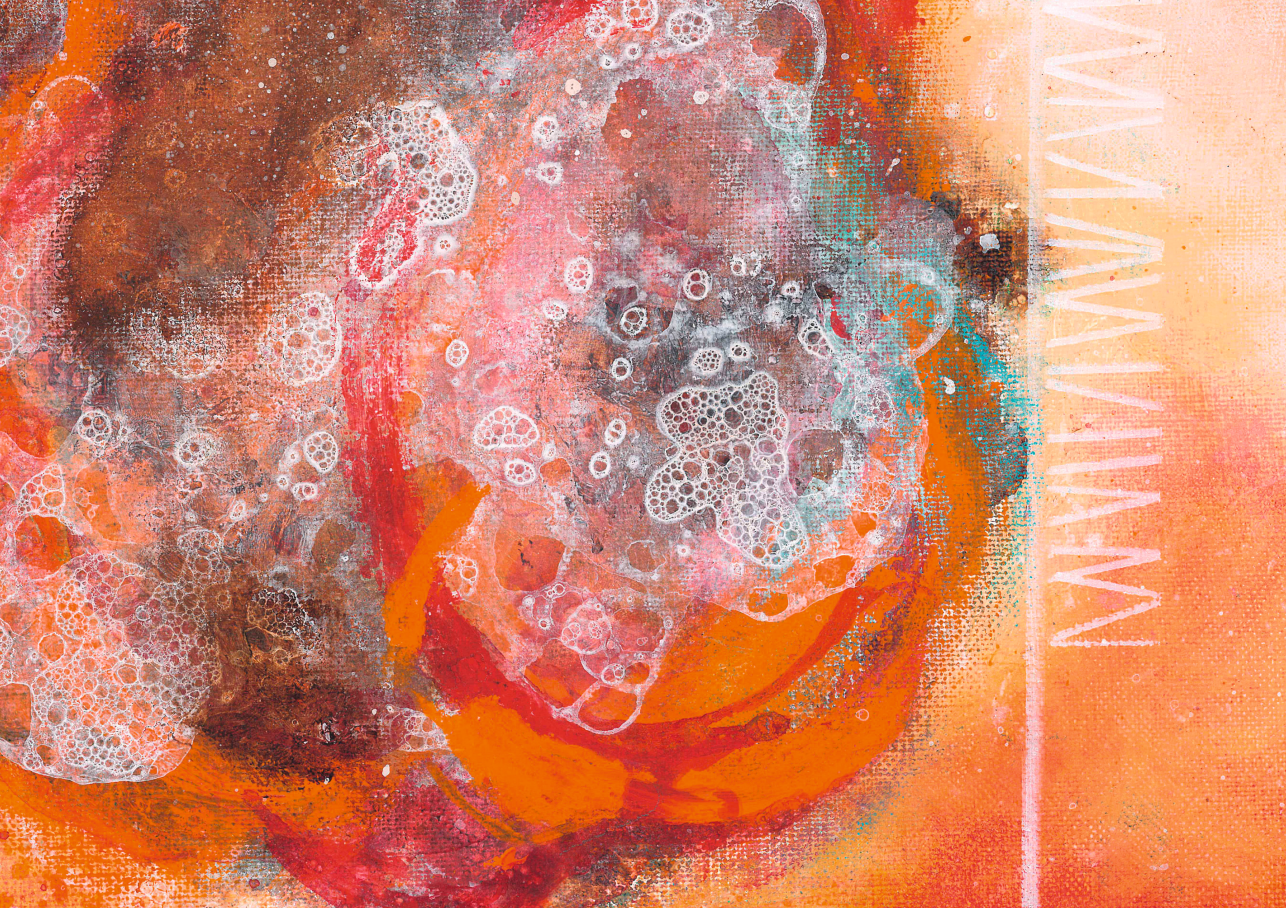

Human iPSC-CMs are gaining prominence as indispensable tools in biomedical and pharmaceutical research, offering key insights into human cardiac physiology and pathology. The increasing relevance of hiPSC-CMs is underscored by their growing market value, dominating the global iPSC market size in 2023 which was valued at USD 1,940.39 million and anticipated to grow to USD 4,355.56 million by 2032 (Polaris Market Research). This surge reflects their expanding applications in safety pharmacology, disease modeling, drug discovery, personalized medicine, and their entry into the clinical research phase for testing heart regeneration therapies. Despite their potential, challenges remain, including the need for better maturation, higher reproducibility, and achieving a balance between complexity, throughput, and cost per datapoint. This discussion integrates insights from the various chapters of this thesis to highlight the current status, challenges, and future directions of hiPSC-CM models in research and development.

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General discussion and future perspectives

The predictive value and regulatory acceptance of hiPSC-CMs in safety pharmacology

The development of hiPSC-CMs represents a significant advancement in translational cardiovascular research, showing great promise for predicting proarrhythmic risk and understanding arrhythmogenic mechanisms. For instance, using beat-to-beat variability of repolarization (BVR) in hiPSC-CMs as a proarrhythmic marker has shown that increased BVR can precede the first arrhythmic event, offering a more specific indicator of arrhythmia inducibility than repolarization prolongation (chapter 3). This finding is particularly relevant for the safety pharmacology and drug development sectors, where reliable prediction of drug-induced arrhythmias is crucial.

Confidence in hiPSC-CMs outperforming conventional cell lines (over-expressing hERG) in predicting drug-induced arrhythmia has grown over the last decade. This increase in confidence can be greatly attributed to international collaborations, such as the CiPA initiative, which launched the first international multisite study to validate the potential of hiPSC-CMs in predicting and classifying drug-induced TdP.[1] Complementary approaches have been undertaken by the Japan iPS Cardiac Safety Assessment (JiCSA)[2] and the Consortium for Safety Assessment using Human iPS cells (CSAHi).[3] These initiatives have played pivotal roles in validating and implementing hiPSC-CMs in cardiac safety pharmacology and provided guidance for the use of hiPSC-CMs in the ICH S7B Q&A guideline for acute proarrhythmia evaluation, marking a crucial step towards regulatory acceptance.[4,5]

In recent years, hiPSC-CM models have demonstrated potential beyond CiPA's focus, enabling risk assessment for non-proarrhythmic cardiotoxicity, including contractile, metabolic, and structural cardiotoxicity. The expanding capabilities of hiPSC-CMs have further motivated the safety pharmacology field to initiate other international public-private partnerships, such as the InPulse CRACK-IT initiative aimed at validating medium-throughput technology platforms to measure the contractility of hiPSC-CMs across 2D and 3D platforms, in which our laboratory took part.[6] Additionally, a follow-up study from CiPA, organized by HESI, evaluated the ability of hiPSC-CM models to detect chronic cardiotoxicity. Initiatives like CiPA, JiCSA, CSAHi, and CRACK-IT highlight the importance of international cooperation in standardizing methodologies and integrating new findings into regulatory frameworks. These collaborations are essential for ensuring the reliability and regulatory acceptance of hiPSC-CM assays in drug discovery and development.

Moreover, the growing potential of human stem cell models, combined with a strong focus on the 3Rs of animal ethics—replacing, reducing, and refining the use of lab animals—has driven regulatory agencies to take meaningful steps to limit the reliance on animal testing. This shift gained significant momentum in 2022 with the passage of the FDA Modernization Act 2.0, which allowed the use of alternative approaches, such as hiPSCs, organoids, and OoCs, to assess drug safety and efficacy.[7] In 2024, the FDA Modernization Act 3.0 was introduced,

aiming to establish clear guidelines and processes to facilitate the adoption of these "new approach methodologies" (NAMs).[8] While the future of drug development may not be entirely animal-free, these regulatory milestones mark a pivotal move towards greater reliance on innovative and human-relevant models.

Challenges and opportunities in mimicking human cardiac function with hiPSC-CMs

Despite promising developments, concerns remain regarding whether hiPSC-CMs express all cardiac ion channels and transporters in proportions that accurately reflect the human heart. For instance, the transient outward potassium current (I_{to}), which plays a crucial role in shaping the early phase of repolarization, appears to contribute minimally to the action potential shape in hiPSC-CMs.[9] Conversely, hiPSC-CMs express ion channels that are absent in the adult human myocardium. As an example, the spontaneous beating and sensitivity of hiPSC-CM beating rate to ivabradine strongly suggest significant activity of the hyperpolarization-activated funny current (I_f) in hiPSC-CMs, similar to what is observed in human sinus node cells, but not in human ventricular CMs.[10]

The less negative diastolic potential of hiPSC-CMs has also attracted much attention, generally assumed to be a marker of immaturity and attributed to abnormally low current densities of the inward rectifier current (I_{K1}). In chapter 3, we addressed this by injecting a simulated virtual I_{K1} through the dynamic-clamp technique, which allowed us to restore a physiological resting membrane potential. However, technical issues should not be overlooked as contributors to the less negative diastolic potential of hiPSC-CMs. It appears that I_{K1} densities are not necessarily too low in hiPSC-CMs. Instead, a combination of the smaller size of hiPSC-CMs compared to adult CMs and the use of patch clamp techniques likely contributes to the less negative potential.[9] In addition, proper cell-cell coupling may be critical for the generation of a correct diastolic potential, making sharp microelectrodes in intact tissue, or technologies capable of measuring the electrophysiology in cell monolayers (e.g. microelectrode array, MEA) favourable.

MEA is a valuable tool for assessing the electrophysiology of hiPSC-CMs at midscale throughput. This was demonstrated throughout this thesis, where it proved its value for various applications: proarrhythmia assessment (chapter 3), rapid screening of acute- (chapter 4) and long-term (chapter 6) drug effects, and compatibility with machine learning approaches to predict ion channel block and TdP risk (chapter 5). MEA can also offer electrical pacing, which has been shown to increase the maturity of hiPSC-CMs when applied over an extended period.[11–13] In addition to electrical pacing, other methods such as prolonging culture time or modulating chemical and mechanical culture conditions can also induce maturation.[14,15] For instance, hormonal signalling and metabolic substrate selection can markedly enhance the maturation of hiPSC-CMs by improving gene expression profiles,

contractility, electrophysiology, and metabolism.[14] Different interventions target distinct facets of maturation, suggesting that activating multiple signalling pathways simultaneously might lead to even greater maturation. To date, methods to model hiPSC-CM cultures in 3D have been arguably the most successful to enhance maturation *in vitro*. 3D models more closely resemble the *in vivo* cardiac environment and therefore provide a more accurate representation of human cardiac physiology.

Apart from cellular immaturity, the interaction of CMs with different cell types in the *in vivo* microenvironment is important to consider. The adult myocardium is not only composed of CMs; in fact, only about 30% of all cells are CMs, while the remaining 70% is a combination of cardiac endothelial cells (cECs), cardiac fibroblasts (cFbs), and vascular stromal cells.[16] Incorporating different cardiac cell types *in vitro* has been shown to increase the maturation state of hiPSC-CMs as well as to reveal cardiac disease phenotypes.[17,18] In chapter 7 we developed 3D cardiac microtissues (cMTs) consisting of approximately 70% hiPSC-CMs, 15% hiPSC-cECs, and 15% hiPSC-cFbs. These cardiac cell types were differentiated using a small-molecule-based protocol newly developed in this thesis, which resulted in increased reproducibility and reduced costs. Instead of using MEA, we employed fluorescence-based methods to assess voltage and calcium kinetics of the cMTs in high-throughput (384-well plates). To validate the model, we conducted a blind screen of inotropic test compounds, including calcium sensitizers and PDE3 inhibitors. While PDE3 is the major isoform restricting adrenergic responses in adult human myocardium, the dominant impact of PDE4 appears to be a peculiarity of hiPSC-CMs, even in 3D models, complicating the detection of some positive inotropic responses. Nevertheless, our study demonstrated that cMTs could detect the effects of challenging compounds, such as levosimendan, highlighting the increased maturation state of cMTs.

The need for standardization and proper validation

Although substantial progress has been made in the structural and functional characterization of hiPSC-CMs, results still vary widely between different studies.[9] These variations can be attributed to methodological issues in recording techniques and biological differences between cell lines, cell batches, and culture techniques (e.g., age of cultures, different culture media). Proper validation and standardization of hiPSC-CM models would lead to less variability and are essential for adoption by the pharmaceutical industry.

A first step towards this goal is implementing thorough quality control (QC) for cell purity and function, which is typically incorporated into the production processes of commercial cell suppliers. Effective QC ensures reproducibility and reduces batch-to-batch variation. Additionally, advancements in cryopreserving hiPSC-derived cardiac derivatives are providing more flexibility and reducing inter-experiment variation. For these reasons, cMTs from cryopreserved, quality controlled hiPSC-derivatives might have an advantage over self-

organizing cardioids from hiPSCs for robust and reproducible drug screening. Cardioids, on the other hand, have the advantage over cMTs to study self-organizing principles of human cardiogenesis.[19] While cryopreservation methods for individual cells have been well established in recent years, progress is needed to develop techniques for cryopreserving whole plates of (3D) cardiac models to make them ready-to-use.

The use of standardized reference drug sets that target multiple mechanisms of cardiac function—such as those affecting contractility suggested in chapter 2 and applied in chapter 7—would be an essential next step for functional validation of novel hiPSC-CM models. Subsequently, integrating automated procedures and robotics would further increase standardization, reduce operator bias, and enhance the scalability of hiPSC-CM models. Chapter 7 incorporates these advancements and presents a significant milestone by describing the automation of cMT formation and maintenance using refined differentiation protocols and robotics. We have shown that utilizing cryopreserved differentiated hiPSC-derived cardiac derivatives, along with robotics and automated techniques, streamlines the process, minimizes variability, and consequently enhances the success rate in a time-efficient manner. Such an approach towards industrialization of 3D cMTs holds significant promise for implementation of hiPSC-CM models within drug discovery programs.

Towards disease modelling and high throughput screening

The unique potential of hiPSC-CMs to mimic key features of original cardiac diseases while offering speed and scalability, have shown promise in overcoming the "translational failure" associated with animal models. However, revealing robust disease-associated phenotypes *in vitro* has been challenging due to the immaturity of the models, low-throughput analytics, and reproducibility issues. This has been evident for modelling the inherited arrhythmogenic disease, CPVT1, *in vitro*. Studies have been able to identify disease-associated features such as stress-induced arrhythmias, but have struggled with limitations of low-throughput 2D models and outputs that exhibit low reproducibility, with success rates around 30%.[20,21] These limitations make the models insufficient for implementation in HTS.

In chapter 7 we achieved a breakthrough in modelling CPVT1 *in vitro* by using CPVT1-MTs pretreated with dbcAMP to reliably trigger CPVT1-associated arrhythmias in a reproducible (success rate around 90%) and high-throughput manner. We have previously shown that dbcAMP increases electrical maturation even in 2D hiPSC-CMs.[17] In this study, we hypothesized that dbcAMP bypasses adrenergic stimulation, increasing intracellular cAMP to much higher levels than typically achieved in hiPSC-CMs after β-adrenergic stimulation alone, likely due to generally low basal receptor tone and low cAMP levels in hiPSC-CMs (chapter 2). This study exemplifies how to leverage the strengths of hiPSC-CMs and adapt to their limitations, making the model fit for purpose.

Using robotics and semi-automated procedures, we subsequently conducted an HTS of 92 test compounds to identify candidates capable of rescuing CPVT1-induced arrhythmia. The HTS demonstrated that the arrhythmic phenotype was rescued in 100% of the cMTs by the reference drug, flecainide. We subsequently identified 17 hits, of which 9 had never been tested in a CPVT1 *in vitro* model before. Secondary screens are planned to confirm the potential of these compounds as anti-arrhythmic drugs for CPVT1. Future studies could examine these hits in CPVT1-MTs derived from patients with different arrhythmogenic phenotypes or varying responsiveness to drugs. If these features can be captured and distinguished in cMTs, it will open new opportunities for patient stratification, personalized treatment, and the discovery of new drugs through HTS.

Future directions

Expecting hiPSC-CM models to be perfect is unrealistic because a model is, by definition, a simplification of reality. It is essential to build on their strengths and accommodate their challenges, particularly incomplete maturation, to make the models fit for purpose. Methodologies that couple advanced hiPSC-CM models with computational modelling, such as the machine learning techniques used in chapter 5 and the digital twin approach used in chapter 6, will maximize the utility of these models. These approaches should aim to balance complexity, throughput, and cost, thereby enhancing the utility of hiPSC-CMs in both primary screens and detailed mechanistic studies. Future studies integrating "*in vitro-in silico* synergy" will likely lead to increased reliability of novel *in vitro* models and subsequent improved translatability to clinical outcomes. Achieving this synergy and advancing hiPSC-CM technology requires collaborative efforts among regulatory agencies, academia, and industry. These international collaborations are needed to agree on validation and standardization strategies for novel *in vitro* models to be widely accepted and implemented within the pharmaceutical industry.

In conclusion, hiPSC-CMs have revolutionized cardiac research, offering powerful tools for (personalized) drug discovery and development. While challenges related to maturation and variability persist, ongoing technological advancements and collaborative efforts aim to overcome these hurdles. The future of hiPSC-CMs lies in their continued integration with cutting-edge technologies and computational models from multiple disciplines. It would be overly simplistic to attribute today's innovative models and technologies to the efforts of a single individual, laboratory, or discipline. To truly advance the goal of better therapeutic strategies and improved patient outcomes, we must continue building bridges across diverse disciplines.

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