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Improving biological tools for disease modelling and drug discovery:

Human iPSC-derived Atrial Cardiomyocytes.

AXOL

Discovery Stems From Here

Improving biological tools for disease modelling and drug discovery: Human iPSC-derived atrial cardiomyocytes.

Introduction

The rapid development of induced pluripotent stem cell-derived cells (iPSC) is set to revolutionise modern drug discovery through the increased utilisation of these human cell reagents in target validation studies, predictive screening assays and other translational phenotypic applications. There remains a challenging gap as new drugs are progressed from traditional *in vitro* screening assays to preclinical *ex vivo* and *in vivo* animal models and eventually into human clinical trials. This lack of human translation applies to both drug discovery and safety pharmacology testing. Axol Bioscience is a leading provider of iPSC-derived cells and reagents designed and validated to deliver more powerful and reliable translational data during the drug discovery process. These tools offer the potential to create more predictive and cost-effective 'disease-in-a-dish' and safety pharmacology 'clinical trial-in-a-dish' applications for academic, biotech and pharmaceutical company clients.

Here we summarise our latest development in cell models, human iPSC-derived atrial cardiomyocytes. Atrial fibrillation is the most common arrhythmia observed in the clinic and accounts for up to 30% of strokes, affecting 6 million patients in Europe and increasing in occurrence up to 25% or more with age, leading to significant morbidity and mortality (Hancox *et al*., 2016). The irregular heartbeat and disturbed electrical activity of patients with atrial fibrillation, is commonly treated by surgical interventions such as pacemakers or ablation of diseased tissue, or treatment with non-selective class IC (Na_v) and III (K_v) ion channel antiarrhythmic drugs which can have serious side-effects (Burashnikov & Antzelevitch, 2010). Thus, new approaches to treat atrial fibrillation are required. Although manifesting as a chronic structural disease, human genetics reveal a role in atrial fibrillation for mutations in ion channel genes such as *KCNQ1* and *KCNEx*, *KCNH2*, *SCN5A*, *KCNJ2*, *KCNN3*, *HCN4* and *KCNA5* (Yang *et al*., 2009; Ellinor *et al*., 2012). The selective expression of certain potassium ion channels in human atria such as $K_v1.5$ (*KCNA5*), $K_{ir}3.1/K_{ir}3.4$ (*KCNJ3/KCNJ5*), and $K_{Ca}2.2$ and $K_{Ca}2.3$ (*KCNN2/3*), and their role in action potential (AP) repolarisation, has rendered them attractive targets for atrial fibrillation drug discovery, leading to clinical trials and marketing of drugs, such as MK-0448, XEN-D0103, AP30663, and Vernakalant by pharmaceutical and biotech companies including Merck, Xention, Acesion, and Cardiome (Pavri *et al*., 2012; Ravens *et al*., 2013; Ford *et al*., 2016).

Considerable effort has been made to understand the cellular mechanisms of atrial fibrillation and to use this knowledge to develop safer and more effective antiarrhythmic drugs (El Haou *et al*., 2015). However, traditional drug discovery approaches employing non-cardiac cells and non-human preclinical animal models of atrial fibrillation may not reliably replicate the physiology of human atrial cardiomyocytes, or predict patient efficacy and safety. This is evidenced by the lack of new atrial fibrillation drugs licensed in recent years. We are the first to develop, validate and commercially release human iPSC-derived atrial cardiomyocytes, which can be used for more reliable modelling of atrial fibrillation and facilitate the discovery and development of more effective treatments for atrial fibrillation.

Creation & characterisation of human iPSC-derived atrial cardiomyocytes

Human iPSC were generated by footprint-free episomal reprogramming of CD34+ cord blood from a healthy, newborn male donor using defined factors OCT3/4, KLF4, SOX2, and c-MYC (Yamanaka factors used in accordance with licensing agreement with iPS Academia Japan). The iPSC were then differentiated to atrial cardiomyocytes using an adaptation of the method in Burridge *et al* (2014) with the addition of specific small molecules at critical time points during differentiation to drive atrial fate. Once produced, the quality of the human iPSC-derived atrial cardiomyocytes was assessed for sterility, viability, culture properties, morphology, beat rate and protein expression to confirm an increased population of atrial phenotype within the cardiomyocyte population.

The successful production of iPSC-derived atrial cardiomyocytes is shown in Fig. A, where immunocytochemistry reveals strong labelling for cardiac and atrial-specific proteins. Cells are richly labelled by antibodies directed against Troponin T and myosin light chain (MLC), confirming they are cardiac myocytes expressing contractile proteins associated with muscle sarcomeres. Their atrial differentiation is indicated by enrichment for atrial over ventricular MLC, and strong labelling for atrial natriuretic peptide (ANP) which is specifically secreted by atrial myocytes upon cell stretching.

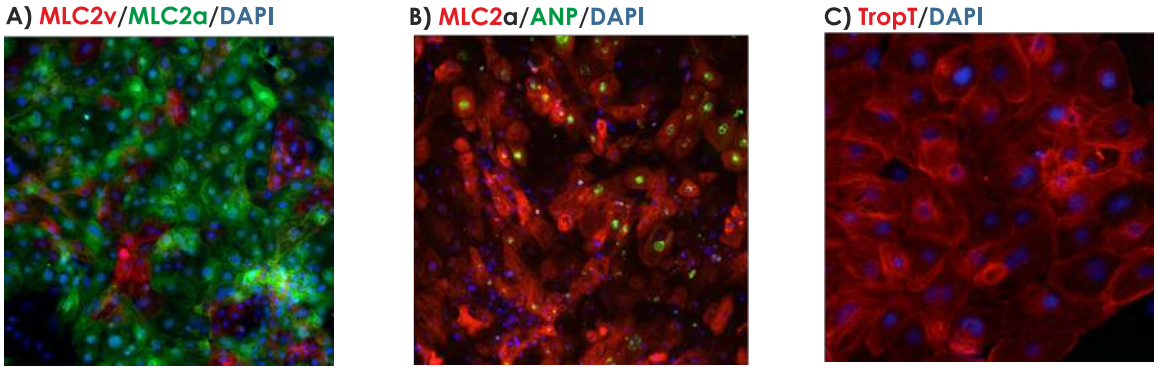


Figure A: Visualisation of cardiac and atrial-specific protein markers in hiPSC-derived atrial cardiomyocytes by immunocytochemistry.
A) The expression of atrial myosin light chain MLC2a (*red*) was higher than that of the ventricular myosin light chain MLC2v (*green*). **B)** Cells positive for MLC2a (*red*) also expressed atrial natriuretic peptide (ANP, *green*). **C)** All cells were positive for the general cardiac marker Troponin T (TropT, *red*).

Validation of human iPSC-derived atrial cardiomyocyte genotype and phenotype

The cardiac genotype of Axol's Human iPSC-derived atrial cardiomyocytes was further characterised at the molecular level using gene chip microarray analysis of cardiac and atrial-specific markers (Fig. B). This data confirms that general cardiac genes, such as the NK homeobox 5 (NKX2.5), ryanodine receptor (RYR2), Phospholamban (PLN), Ca^{2+} ATPase (ATP2A2), and the contractile proteins TNNC1, TNNT2, TNNI1 and ACTN2 are upregulated in the iPSC-derived atrial cardiomyocytes compared with their iPSC progenitors. Atrial-specific genes, such as NR2F1, NR2F2 (COUP-TF), NPPA (Atrial Natriuretic Factor), MYL7 (Myosin Light Chain MLC2a), Visinin-like calcium binding protein 1 (VSNL1) and Connexin 40 (GJA5) are significantly upregulated in the iPSC-derived atrial cardiomyocytes compared to iPSC progenitors and iPSC-derived ventricular cardiomyocytes from the same donor. Significantly, the expression of atrial-specific ion channel genes *KCNA5* (K_v1.5, IK_{ur}) and *KCNJ3* and *KCNJ5* (K_{ir}3.1/3.4; I_{KACh}) are also selectively increased in the iPSC-derived atrial cardiomyocytes compared with iPSC-derived ventricular cardiomyocytes, suggesting that they manifest the correct biophysics and pharmacology for atrial fibrillation drug discovery and disease modelling (which we

confirm below). In contrast, ventricular-specific genes such as *HEY2*, *GJA1*, *ACE2* and several types of collagen are down-regulated in the iPSC-derived atrial cardiomyocytes. This genotype profile is consistent with publications on gene and mRNA expression in Embryonic Stem Cell (ES)-derived atrial cardiomyocytes (Devalla *et al*., 2015, Laksman *et al*., 2018), confirming the creation of differentiated iPSC-derived atrial cardiomyocytes.

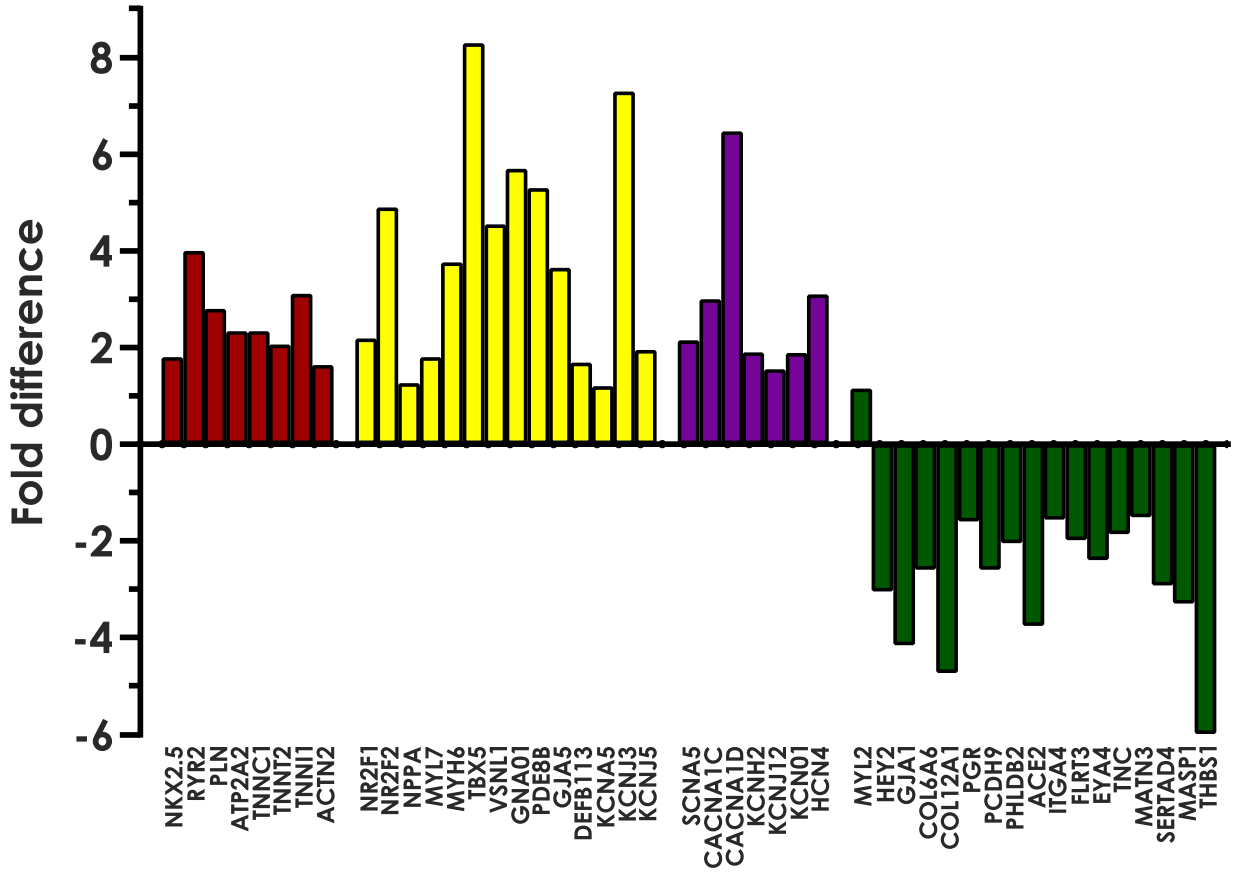
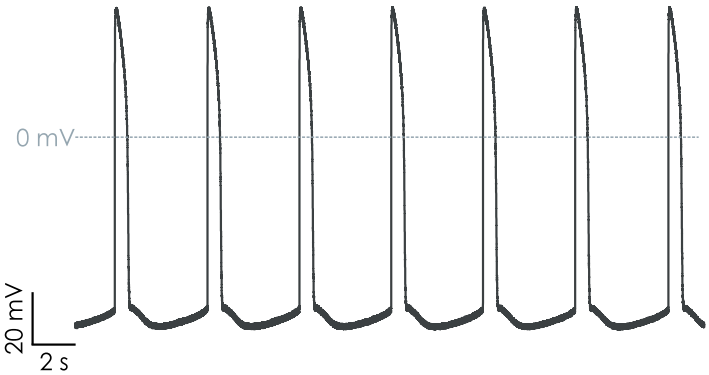


Figure B: Presence of cardiac and atrial-specific cardiomyocyte genes.
Fold difference in gene chip microarray expression for cardiac genes (red), atrial-specific genes (yellow), cardiac ion channels (purple), and ventricular-specific genes (green), compared with signals detected in iPSC-derived ventricular cardiomyocytes. Note that *KCNA5*, *KCJN3* and *KCNJ5* genes are atrial-specific ion channels.

As ion channels and electrical signalling are fundamental aspects of healthy atrial cardiomyocyte function and atrial fibrillation disease processes, we also undertook biophysical and pharmacological validation of the iPSC-derived atrial cardiomyocytes using manual patch clamp electrophysiology recordings. Firstly, the biophysical features of the iPSC-derived atrial cardiomyocytes were determined using current clamp recordings of AP parameters (Fig. C). At room temperature, the cells spontaneously beat and fired APs with a frequency of 0.3 Hz, which was facilitated by a negative resting potential of -73 mV and a robust upstroke velocity - indicative of good $Na_v1.5$ channel availability - yielding strongly overshooting AP amplitudes. Similar to native tissue from human cardiac chambers, Axol's human iPSC-derived atrial cardiomyocytes had shorter AP durations than Axol's human iPSC-derived ventricular cardiomyocytes from the same donor (e.g. APD90 at room temperature of 419 vs 533 ms, respectively, N>30 cells each).



AP parameter	Mean Data (N = 31)
MDP (mV)	-73.6 ± 0.8
dv/dt_{max} (V/s)	44.6 ± 3.4
APA (mV)	-120.2 ± 1.0
APD20 (ms)	252.4 ± 6.3
APD50 (ms)	338.0 ± 6.7
APD90 (ms)	419.0 ± 7.0
Frequency (Hz)	0.28 ± 0.01

Figure C: Biophysical characteristics of spontaneous iPSC-derived atrial cardiac APs. Manual patch current clamp recordings reveal spontaneous firing of overshooting APs from a negative resting maximal diastolic potential (MDP), with a rapid upstroke velocity (dV/dt) and relatively short AP duration (APD).

We confirmed the functional expression of relevant cardiac ion channels using selective pharmacological ligands. A core panel of $Na_v1.5$, $Ca_v1.2$ and hERG channels, which was shown to be expressed in the iPSC-derived atrial cardiomyocytes by gene microarray, was confirmed by the effects of the selective inhibitors Lidocaine, Nifedipine and E-4031 respectively (Fig. D). These agents produced the expected changes in AP upstroke and amplitude ($Na_v1.5$), APD20 ($Ca_v1.2$), and APD90 (hERG), which highlights the functionality of these ion channels. Furthermore, when applied to spontaneously beating iPSC-derived atrial cardiomyocytes, moderate concentrations of the hERG blocker, Dofetilide, induced early after depolarisations, consistent with native human cardiomyocytes.

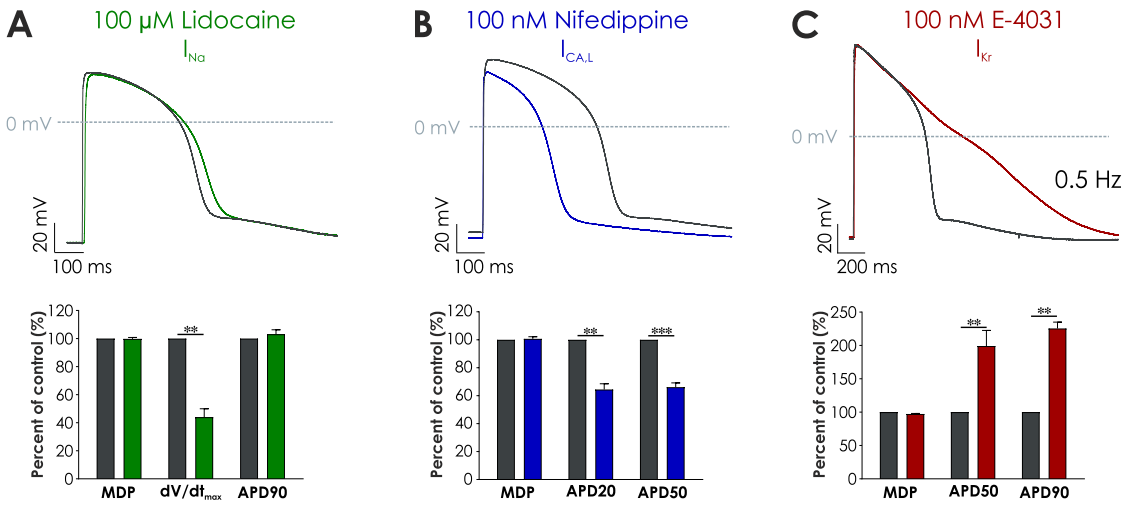


Figure D: Core cardiac ion channel pharmacology of iPSC-derived atrial cardiomyocytes. Manual patch current clamp recordings of evoked APs (1 Hz stimulation, except E-4031 at 0.5 Hz) reveal characteristic effects of selective $Na_v1.5$ (100 µM Lidocaine, **A**), $Ca_v1.2$ (100 nM Nifedipine, **B**) and hERG channel inhibitors (100 nM E-4031, **C**) on upstroke velocity, intermediate (APD20-APD50) and late phase repolarisation duration (APD50-APD90) respectively. Statistical significance using paired Student's t-test; ** P<0.01, *** P<0.001.

Finally, the atrial phenotype of these iPSC-derived cardiomyocytes was confirmed by the characteristic effects of modulators of atrial-specific ionic currents shown to be upregulated in the gene microarray data. Activation of the acetylcholine-activated inward-rectifying potassium current (I_{KACh}) mimicked the negative chronotropic effect of vagal tone to slow spontaneous activity, whilst inhibition of the ultra-rapid delayed rectifier potassium current (I_{Kur}) prolonged APD20 (Fig. E). Thus, the functional ion channel repertoire of these human iPSC-derived atrial cardiomyocytes recapitulates that seen in native human tissue, and reveals the

expression of ion channel targets relevant to atrial fibrillation drug discovery (El Haou *et al.*, 2015).

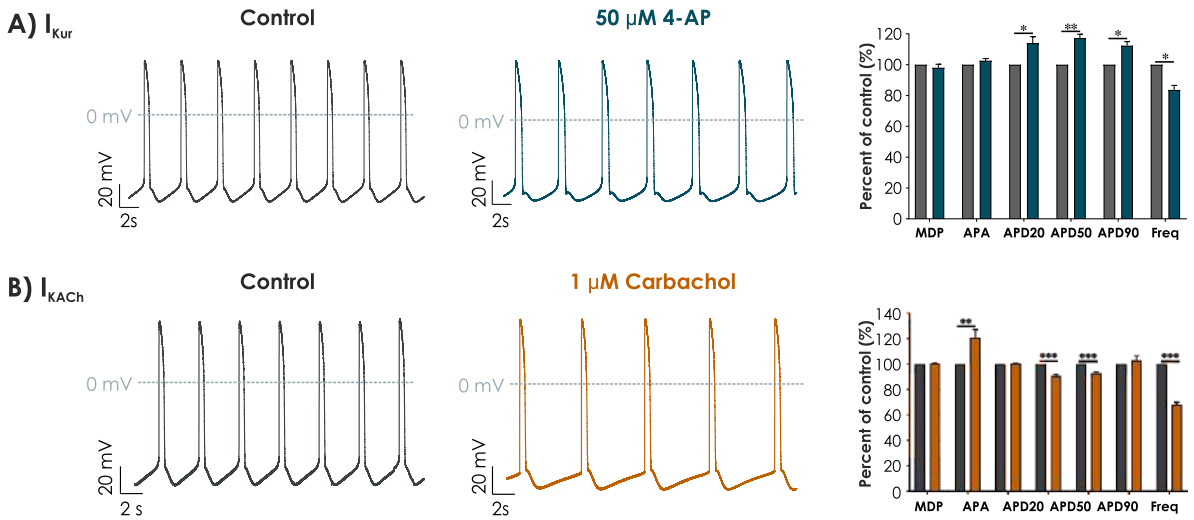


Figure E: Atrial-specific ion channel pharmacology.
Manual patch current clamp recordings of spontaneous APs reveal characteristic effects of selective modulators of IK_{ur} (50 µM 4-AP, **A**) and I_{KACh} channels (1 µM Carbachol, **B**) known to be selectively expressed in human atria. 4-AP slows AP repolarisation and firing rate, while I_{KACh} activation shortens APD but also profoundly slows AP firing rate. Statistical significance using paired Student's t-test; * P<0.05, ** P<0.01, *** P<0.001.

The biophysical and pharmacological profile of Axol's human iPSC-derived atrial cardiomyocytes were found to be very consistent when we compared data obtained from initial pilot batches and final commercial scale-up material. Such batch-to-batch consistency is essential in cell reagents and assay applications that will be used for drug discovery screening and disease modelling.

Our collaborative validation efforts demonstrate the commitment of Axol Bioscience as an iPSC supplier and Metrion Biosciences as a CRO assay service provider to deliver robust and predictive iPSC-based reagents and assays for our customers and clients in academia, biotech and pharmaceutical sectors. Human iPSC-derived cells can be challenging to produce and customers should expect providers to offer consistent and scalable supply with well-established commercial freedom-to-operate; challenges that are recognised and addressed by Axol Bioscience. Similarly, the full integration of commercial iPSC-derived cardiomyocytes as models of human cardiomyocytes and their use in drug discovery programs relies on reproducible and validated electrophysiological and pharmacological readouts. Such data can be delivered by specialised and experienced CRO providers, such as Metrion Biosciences, through their expertise in compound handling, ion channel function, iPSC-derived cardiomyocyte screening assays and data interpretation. Together, our characterization data indicates that iPSC-derived atrial cardiomyocytes provide a cost-effective and consistent assay for building relevant *in vitro* models of atrial fibrillation.

Advantages of Axol's human iPSC-derived atrial cardiomyocytes

There are several compelling reasons why these human iPSC-derived atrial cardiomyocytes represent a useful model system for improving atrial fibrillation drug discovery and disease modelling.

- ***Ease of use***

Compared to the complexities and challenges in sourcing, extracting and culturing native human atrial cardiomyocytes, human iPSC atrial cardiomyocytes offer far greater utility. Their source is reliable, thanks to the delivery of fresh or storage of frozen cells, with the option to obtain larger quantities of material from the same iPSC differentiation batch. Consistent supply also facilitates better planning and execution of experiments, and delivery of important screening data to tight timelines. The robust phenotype of these cells allows complex experiments to be undertaken by experienced staff to align iPSC data with native tissue values, but in addition more traditional screening experiments can be carried out by staff using industry-standard plate-based assay platforms.

- ***Consistency***

The variability in native human cardiomyocyte data is considerable and, as a consequence, larger samples are required to reveal significant treatment effects. This variability results from intra- and inter-patient differences, and the effects of harsh isolation techniques on delicate cells and tissues. In contrast, much more consistent data can be obtained from *in vitro* iPSC-derived atrial cardiomyocyte assays, reducing costs and data delivery timelines whilst improving data quality and predictive power. Axol's iPSC-derived atrial cardiomyocytes are generated from a young, healthy donor, which is not always the case when native human atrial cells are extracted from atrial fibrillation patients who tend to be older and present with fibrous atrial appendages. Many of these patients are suffering from chronic disease and have been exposed to multiple drug treatments, further increasing the variability of their "baseline" data prior to compound screening.

- ***Translatability***

A major aim in the development of iPSC technology is to create accessible cell and assay reagents that more faithfully reflect native human cells and tissues, and thereby deliver more reliable models of function and disease than current *in vitro*, *ex vivo* and *in vivo* systems based on non-human animal species. This is particularly important for human disease modelling and drug discovery efforts, where current levels of translation from test systems to human patients are in urgent need of improvement to increase efficiency and predictivity, and thus reduce costs and drug side-effects. Here we have shown that the key genotypic and phenotypic features of Axol's iPSC-derived atrial cardiomyocytes make them a suitable *in vitro* translational system for cost-effective and reliable atrial fibrillation disease modelling and ion channel target drug discovery.

- ***Future uses***

We envisage these iPSC-derived atrial cardiomyocytes will be amenable to a number of different assay formats applied to disease modelling and drug discovery purposes. In 2D cultures we expect robust signals in plate-based screening assays employing electrophysiology readouts such as multi-electrode array (MEA) and impedance measurements of excitability, field potential duration (FPD), and contractility. Functional imaging should also be possible using Ca^{2+} and voltage-sensing dyes, as well as optogenetic actuators and reporters; contractility could also be assessed using optical imaging techniques. Axol's human iPSC-derived atrial cardiomyocytes should also be suitable for genotypic and phenotypic screening on

industry-standard high content analysis (HCA), metabolic signalling, and gene reporter assay platforms.

We imagine that many of these 2D assay applications may also be adapted to 3D iPSC-derived cardiomyocyte cultures, allowing the creation of 'mini atria' or engineered heart tissue (EHT) from purified atrial cardiomyocyte populations, and development of more complex organoids and 'mini hearts' using mixtures of sino-atrial, atrial, and ventricular cardiomyocytes and cardiac fibroblast iPSCs. These iPSC models would be particularly useful for understanding human cardiac development and function and disease processes, as well as providing more complex translational systems for drug discovery purposes.

The faithful recapitulation of native human atrial genotype and phenotype in this iPSC cell reagent suggests it will have great utility for atrial fibrillation disease modelling. We imagine that some creative disease-in-a-dish applications may be developed, such as emulating the current drug discovery model of tachypaced dogs by employing electrical or optogenetic pacing of iPSC-derived atrial cardiomyocytes to induce structural and gene expression changes and excitability remodelling typical of human atrial fibrillation disease.

Finally, application of genetic engineering techniques to these iPSC-derived cardiomyocytes may allow development of personalised patient-specific disease models, expressing rare mutations in cardiac ion channels and other proteins previously identified in atrial fibrillation patients.

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Notes

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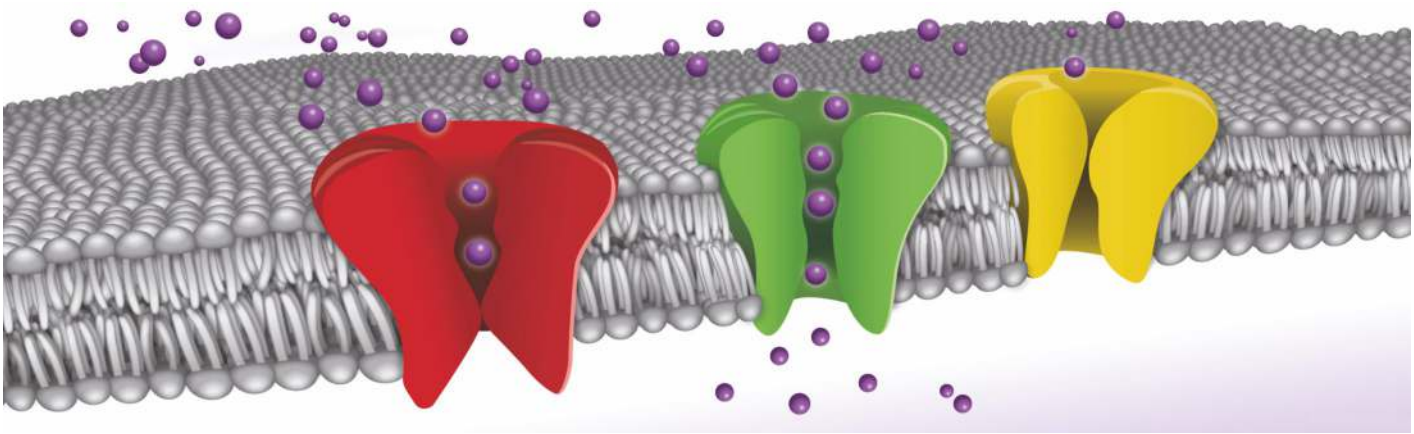

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