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LETTER TO THE EDITOR

Conditional immortalization of human cardiomyocytes for translational *in vitro* modelling of cardiovascular disease

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The increasing prevalence of atrial fibrillation (AF), its high socio-economic burden and its suboptimal treatment options[1] make it an important focus area of cardiovascular research. Where traditionally animal-based models have been used to improve mechanistic insight and to develop novel therapeutics, differences in (patho)physiology between humans and animals, combined with increasing public opposition to animal testing, have resulted in a shift towards disease models based on human cells.

The recently published consensus document of the ESC Working Group on Myocardial Function and the ESC Working Group on Cellular Biology of the Heart[2] provides an extensive overview of animal-based disease models and animal-free innovations across cardiovascular research. Currently, animal-based AF research models ranges from fruit flies and zebrafish, to rodents and large domestic mammals, with the latter species showing the closest (although still limited) resemblance to human (patho)physiology. Even though the general consensus is that these animal models (at least on the short-term) will maintain an important place in preclinical research, there is a need for more clinically relevant and standardized human *in vitro* models of AF. Current sources of well-differentiated human atrial myocytes (AMs) for such models are, however, limited. The poor availability of human atrial tissue, combined with the tendency of human adult cardiomyocytes to dedifferentiate in culture, makes primary human AMs unsuitable. As highlighted in the consensus document, advances in human pluripotent stem cell (hPSC) differentiation protocols have provided an alternative source of human AMs for *in vitro* modelling. However, in spite of their attractive features and unique applications, current hPSC-derived AMs (hPSC-AMs) are associated with laborious workflows, high phenotypic variation, and overall immaturity.

Coinciding with the publication of the consensus document, our group published a method for conditional cell immortalization, which we applied to create a new robust and scalable source of human AMs for *in vitro* research.[3] In brief, through conditional immortalization based on doxycycline-controlled expression of the early gene products of simian virus 40, human fetal AMs could be expanded at least one-quadrillion-fold and allowed to re-differentiate into fully functional AMs in a tightly controllable manner. The molecular, cellular, and electrophysiological properties of these cells, which were named hiAMs (human immortalized atrial myocytes), closely resembled those of primary AMs. Noteworthy, these hiAMs displayed greater overall transcriptomic maturity and a more mature electrophysiological phenotype compared with hPSC-AMs.

Attempts to create *in vitro* models of human AF using hPSC-AMs have been previously reported.[4,5] These models, however, were unable to represent the rapid activation frequencies (6–8 Hz[6]) seen during human AF. In our recent study,[3] we repeated these attempts using hPSC-AMs, resulting in the same conclusion. Through the development of the hiAM lines, we have been able to take a significant step forward in human AF modelling mainly due to two advantageous properties of the hiAMs. First, the more mature electrophysiological phenotype of cardiomyogenically differentiated hiAM monolayers (with the >10-fold higher conduction velocity compared with hPSC-AM monolayers being the most notable) better approximates the electrophysiological properties of patients' tissue. Second, the hiAM lines can easily produce the massive numbers of fully functional AMs needed for large format cultures, whereas the limited yields associated with the strenuous procedure to generate pure hPSC-AM populations practically restricts their application to small-sized models (*e.g.* 1 cm^2/48-well formats). As re-entrant circuits in patients with AF generally occupy >3 cm^2 of atrial tissue,[7] larger culture formats are a prerequisite for creating representative human AF models. With the advances offered by hiAMs, we were able to create 10 cm^2 confluent cell layers presenting activation frequencies of 7–8 Hz after induction of fibrillatory activity, which is consistent with the clinical manifestation of AF. Not only did these hiAM layers better recapitulate the dynamics of human AF, their arrhythmic activity could be terminated by infusion of anti-arrhythmic drugs commonly used in clinical practice, which was not possible in hPSC-AM cell layers[4].

The consensus document notes that a cell-based model of paroxysmal AF has not yet been developed. In fact, one might actually argue that is remains to be determined in general how to define the different types of AF in the *in vitro* setting. The current clinical definitions focusing on duration (*e.g.* a 1 week cut-off to discriminate between paroxysmal and persistent) cannot easily be applied to *in vitro* models for technical reasons. In the current *in vitro* models of AF, including those based on

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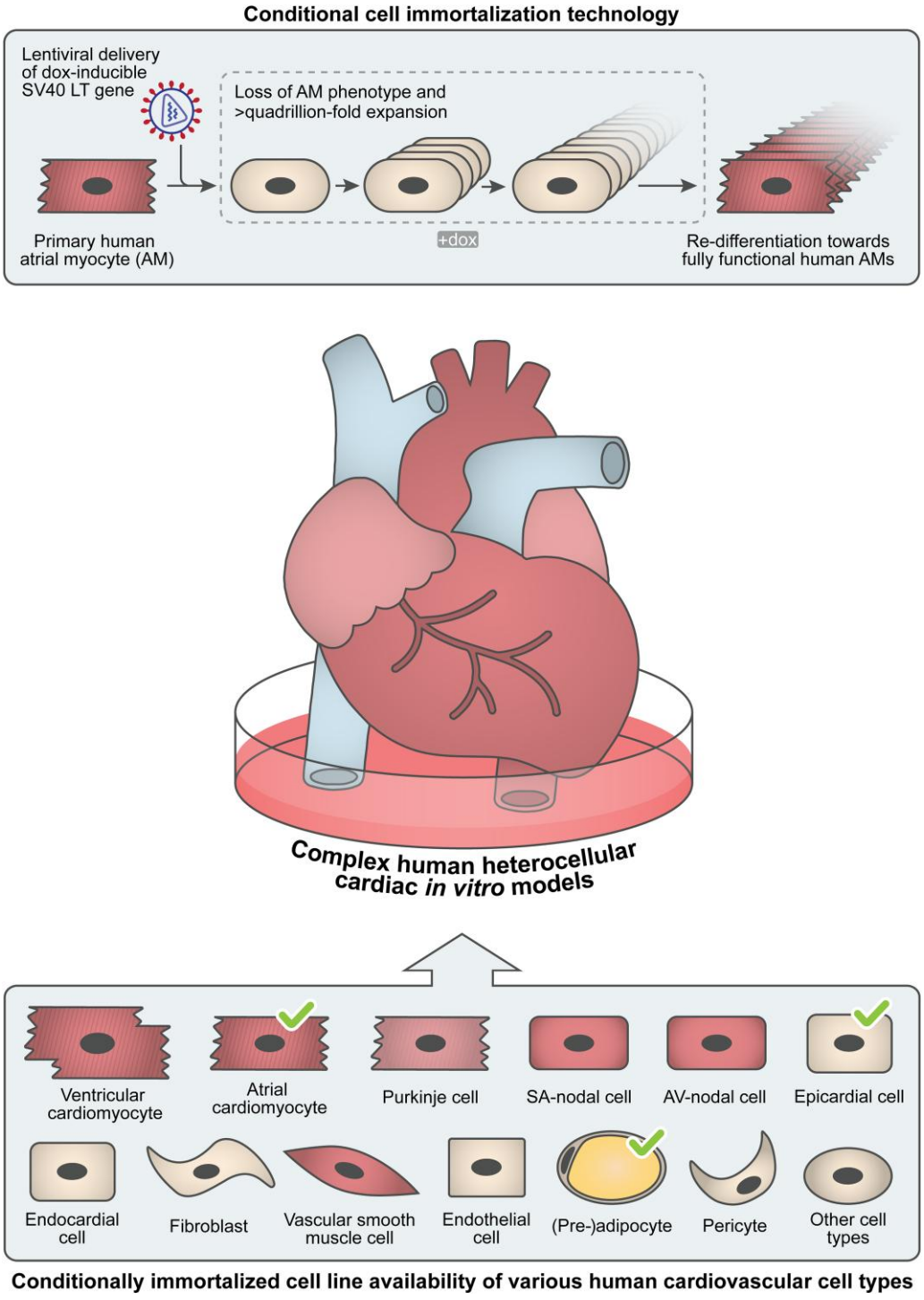

Figure 1 Upper panel, schematic overview of the generation of conditionally immortalized human atrial myocytes (AMs) by transduction of primary human AMs with a lentiviral vector conferring doxycycline (dox)-inducible expression of the simian virus 40 (SV40) large T (LT) gene. Lower panel, generation of human cardiovascular cell types using conditional immortalization technology for the creation of complex human heterocellular cardiac models. Currently, conditionally immortalized lines of human atrial cardiomyocytes,[3] human epicardial cells,[9] and human preadipocytes[10] have been generated.

hiAMs, fibrillatory activity typically terminates spontaneously after a few seconds or continues for a yet to be determined period. Another aspect that requires attention is the perpetuation of fibrillatory activity, which is sustained by re-entrant waves, including spiral waves, in current *in vitro* AF models. However, for the different types of human AF, the actual drivers remain to be fully determined, although other possibilities than re-entry have been proposed.[8] Nevertheless, considering the typical activation frequencies of human AF and the known effects of clinical drugs on its perpetuation, we argue that we have created a clinically relevant human *in vitro* model of AF. Still, there is certainly a need for further

improvement to maximize the relevance and translational value of human *in vitro* models of AF and other rhythm disorders.

The consensus document concludes that novel 2D and 3D *in vitro* technologies will certainly result in a reduction in the number of laboratory animals by the creation of models better capturing human pathophysiology. In this context, it is noteworthy that the distinctive features of hiAMs make them not only highly suitable for arrhythmia modelling but could also facilitate the development of novel disease models in other areas of cardiovascular research. In addition, the conditional immortalization technology at the heart of the hiAMs could be used to generate functional cell lines from other human cardiovascular cells. Together with (future) advances in 3D tissue engineering technology as described in the consensus document, this could help pave the way towards the creation of complex human heterocellular cardiac models with high translational value (*Figure 1*).

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Conflict of interest: D.A.P. and A.A.F.d.V. are inventors of a patent application (US16/480,280) related to this work.

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