STEM CELL CULTURE

Cheaper and less variable expansion

Substituting growth factors with small molecules in the culture medium for the expansion of human pluripotent stem cells reduces costs and lot-to-lot variability.

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Because of their hallmark ability for indefinite self-renewal and their potential to generate any cell type in the adult body, human pluripotent stem cells (hPSCs) serve as an unlimited source of cells for a range of biomedical applications, including cell-replacement therapy, drug screening, in vitro organogenesis and disease modelling. To facilitate such applications, resource-efficient, scalable and reproducible culture methods that maintain high stem-cell proliferation rates, pluripotency and genomic stability for extended hPSC propagation are necessary. To address these needs, defined culture conditions are optimal. Accordingly, over the past 18 years, stem-cell culture conditions have progressively evolved towards chemically defined media and substrates[1]. However, even recombinant protein or protein-derived components of defined culture systems can introduce lot-to-lot variability, and their economics can render large-scale stem cell culture challenging. Reporting in *Nature Biomedical Engineering*, Kouichi Hasegawa and colleagues now show that fibroblast growth factor (FGF) and transforming growth factor beta (TGFβ) — two recombinant protein components present in many standard defined media for the culture of hPSCs — can be replaced with small molecules that are cheaper and less variable[2].

Media for hPSC culture rely extensively on the FGF/TGFβ pathways to maintain pluripotency. Yet Hasegawa and colleagues had previously demonstrated pluripotency maintenance by stimulating Wnt signalling[3]. Notably, Wnt and its downstream effectors play a myriad of context-dependent roles in hPSC biology, including pluripotency and differentiation[4]. To inhibit Wnt-mediated differentiation in their hPSC culture system, Hasegawa and co-authors reported the use of ID-8, a small-molecule inhibitor of the dual-specificity tyrosine phosphorylation-regulated kinase (DYRK)[3]. Building on this progress towards a defined, protein-free medium formulation, the authors have now replaced Wnt3a with 1-azakenpaullone, a small-molecule inhibitor of the glycogen synthase kinase GSK3β. The combination of these two compounds (AKI condition) maintained long-term hPSC pluripotency. The authors then used several additional DYRK inhibitors to further establish that DYRK inhibition can prevent Wnt-mediated hPSC differentiation, although the precise mechanism by which blocking DYRK may inhibit differentiation is unclear (Fig. 1a). Additionally, the particular means used to activate the Wnt pathway likely influences whether DYRK inhibition prevents Wnt-mediated hPSC differentiation. In fact, the authors show that an alternate GSK3β inhibitor (CHIR99021) resulted in gradual differentiation even in the presence of DYRK inhibition. Future mechanistic investigations will be needed to elucidate the rich underlying biological processes involved in AKI-mediated maintenance of pluripotency.

Although ID-8-mediated DYRK inhibition maintained hPSC pluripotency, it also reduced cell-proliferation rates[3], rendering the AKI medium less suitable for effective hPSC expansion. Yet Hasegawa and co-authors observed that ID-8 treatment also increased the expression of the nuclear factor of activated T cells (NFAT)c1, among several targets downstream of DYRK, and that, interestingly, an inhibitor of NFATc1, tacrolimus, increased the proliferation rate and thus supported rapid, long-term expansion of several hPSC lines in AKI. The calcineurin-NFAT pathway has been previously implicated in mouse embryonic stem cell differentiation[5], and in human adult skin stem cells NFATc1 regulates stem cell quiescence (its inhibition leads to increased stem cell proliferation[6]). However, a NFATc1 role in hPSC proliferation is not well understood; mechanistic studies of tacrolimus-mediated increase of hPSC proliferation should thus be revealing.

In addition to effective culture media, culture substrates and passaging approaches also influence the efficiency of stem cell expansion. Ideal substrates are economical, reproducible and scalable, and effective passaging protocols maintain high cell viability and pluripotency. Hasegawa and co-authors also show that the combination of AKI and tacrolimus (AKIT) is compatible with several commonly used substrates and passaging methods. Among the substrates tested, they observed compatibility with Synthemax, a fully synthetic material that is particularly amenable to reproducible hPSC culture. The authors also show that AKIT maintained pluripotency in several hPSC lines for over 30 passages. Another requirement for the long-term propagation of hPSCs is the maintenance of genomic stability, as abnormalities in chromosome numbers and tumorigenic mutations can be favoured during expansion under stressful culture conditions[7]. By using comparative copy number variation (CNV) and loss of heterozygosity (LOH) analyses, the authors found that AKIT-cultured hPSCs were genomically stable and that mutations in the *TP53* gene, which are commonly observed in long-term hPSC culture[7], were not present.

Culture conditions can profoundly affect the cell state of hPSCs (cell states have varying potential for proliferation and differentiation)[8]. By using genome-wide, bulk RNA sequencing (which can broadly assess cell states), Hasegawa and colleagues show that hPSCs cultured in AKIT and in common culture systems exhibited significant differences. On analysing the differentially expressed genes, the researchers attributed such differences to the variable regulation of metabolic processes. Interestingly, metabolic states are markedly different between naive and primed hPSCs, the two commonly accepted stem cell states[8]; however, further experimental investigation into hPSC metabolism did not definitively establish AKIT-cultured hPSCs as either naive or primed. Future work may further explore how AKIT modulates the metabolic or functional states of hPSCs, and whether cells propagated under such conditions are capable of undergoing differentiation into a range of therapeutically relevant cell lineages for biomedical applications.

The AKIT medium developed by Hasegawa and colleagues is more economical (Fig. 1b), and arguably less

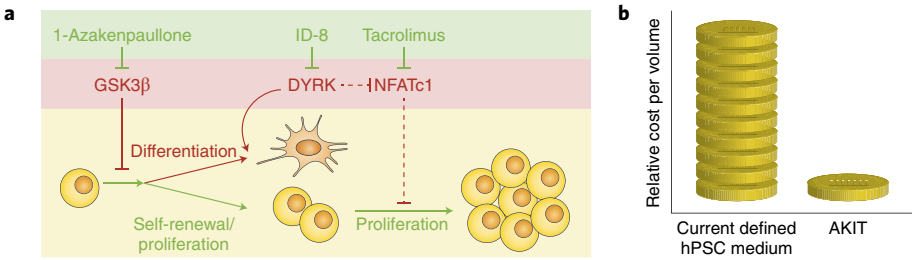

Fig. 1 | A cost-effective and reproducible medium for the culture of hPSCs. a, Potential mechanism of pluripotency maintenance using the AKIT medium. Components or processes in red are inhibited; components and processes in green are active. Solid arrows indicate verified mechanisms; dashed arrows indicate likely, yet currently unproven, roles. **b**, Per unit volume, the AKIT medium costs ten times less than most of the currently used media for chemically defined hPSC culture.

variable, than many commonly used defined culture media. Replacing protein components in hPSC media with small-molecule alternatives may improve the stability of the media, which would have important implications for the increasingly resource-efficient culture of stem cells. This progress may ultimately facilitate hPSC-based research and applications at multiple scales, from small academic labs to industrial cell factories. In addition to offering future directions in elucidating the roles of key signalling factors (such as GSK3β, DYRK and NFATc1) in hPSC pluripotency and in investigating the AKIT-mediated naive versus primed hPSC state, Hasegawa and colleagues' work highlights several new avenues of research. For example, using high-throughput screening to investigate the performance of AKIT-cultured hPSCs on additional synthetic substrates may identify fully defined culture conditions for the rapid, resource-efficient expansion of hPSCs. Furthermore, future studies investigating the compatibility of AKIT with fully defined 3D culture systems may further demonstrate the potential for scalability, because in large-scale hPSC expansion, 3D culture systems arguably provide several benefits over 2D systems. Overall, Hasegawa and colleagues' work represents a significant step forward in the progress towards chemically defined, cost-effective and reproducible hPSC culture systems, and holds promise in the scalable expansion of hPSCs for use in a broad range of biomedical applications. ❐

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