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Expansion of human pluripotent stem cells

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Large numbers of human pluripotent stem cells (hPSCs) are needed to meet the high demands of a range of biomedical applications, including cell replacement therapies and drug screening. Recent advances in media formulations and cell culture platforms have addressed many previous challenges that have hindered efficient expansion. Understanding and addressing the remaining challenges will further facilitate the development of technologies for large-scale hPSC expansion and, to an increasing extent, differentiation.

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Introduction

Human embryonic or induced pluripotent stem cells (hESCs or hiPSCs, respectively) have the potential for indefinite expansion and may thus represent a virtually unlimited cell source for biomedical applications including regenerative medicine [1,2], disease modeling [3], pharmacology and toxicology screening [3,4], and ex vivo organogenesis [5]. However, large numbers of cells are typically required for many of these applications. For example, cell replacement therapies for Parkinson's disease (PD), myocardial infarction (MI), and diabetes I typically would likely necessitate $\sim 10^5$–10^9 cells to survive post-transplantation for each patient [6–8], which combined with 10–100 million patient populations for these diseases [9,10], low target cell differentiation efficiencies, and poor post-transplantation cell survival require $\sim 10^{12-16}$ cells to be generated for each of these indications. Similarly, the *ex vivo* generation of a human liver or heart entails $\sim 10^{10}$ cells [11]. Additionally, $\sim 10^{10}$ cells are required for a typical pharmacological or toxicological screens of $\sim 10^6$ candidates [12]. Therefore, there is a crucial need for scalable culture platforms that can effectively generate large numbers of human pluripotent stem cells (hPSCs). In this review, we compare enabling technologies for large-scale generation of hPSCs, with a focus on recent culture platform (specifically 2D substrates, 3D microcarrier, suspension, and microencapsulation cultures) advances. We highlight the relative strengths and shortcomings of each type of platform and discuss remaining challenges for optimal hPSC expansion.

Advances in culture technologies

Desirable culture conditions for stem cell expansion

Stem cell fate is strongly regulated by environmental factors [13], which for a cell culture system include both culture medium and platform, and it is thus crucial to design, optimize, monitor, and maintain culture conditions for effective hPSC expansion. In general, culture systems for the efficient, scalable, and reproducible expansion of hPSCs should be: fully defined, xeno and feeder-free, good manufacturing practice (GMP)-compatible, cost-effective, scalable and amenable to automation. Additionally culture systems should: generate high yields of pluripotent cells with rapid expansion rates, facilitate high viability passaging and cell harvest, achieve homogenous cell growth and, maintain genomic stability and avoid karyotypic abnormalities, critical for downstream applications.

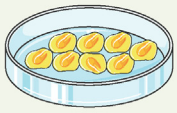
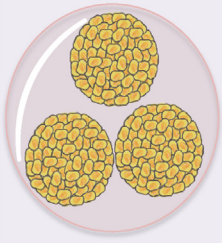
Below, we systematically list the most common types of current culture media and platforms for hPSC expansion, present the most recent developments of each, and discuss their features in light of the optimal criteria for hPSC expansion listed above (Figure 1a and b).

Culture medium

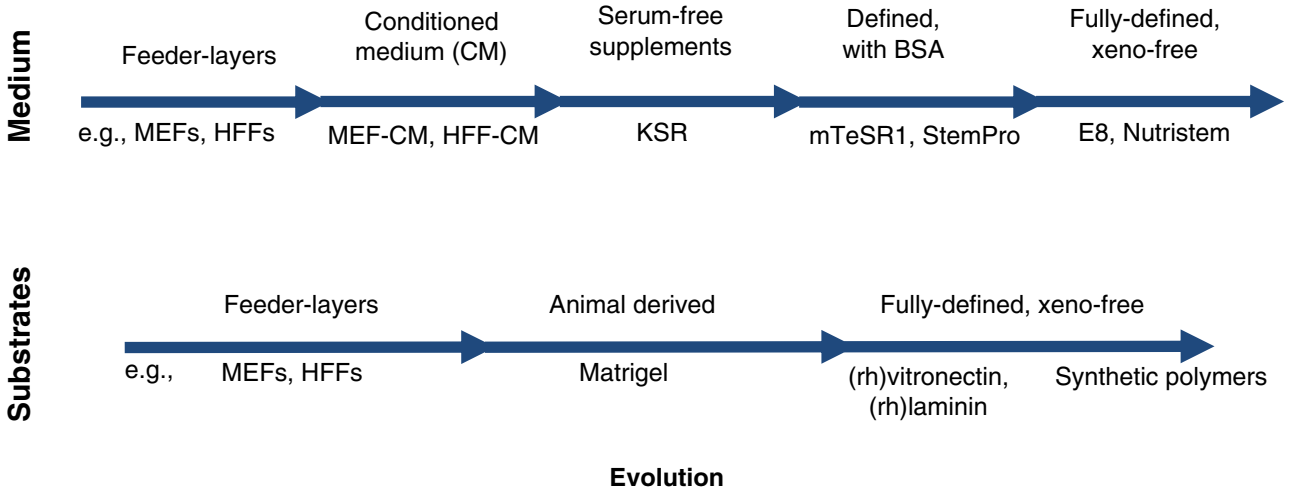
For an in depth analysis of hPSC culture media compositions, the reader is referred to several excellent and comprehensive reviews [14,15]. Briefly, however, hPSCs were initially co-cultured in serum-containing media with feeder cell layers such as murine embryonic fibroblasts (MEFs) that, through secreted and contact mediated factors, offered necessary cues to support hPSC growth [16] (Figure 1b). While the initial hESC derivation was a landmark advance for stem cell biology and regenerative medicine, serum and feeder layers do not meet most of the desirable culture conditions listed in the '*Desirable culture conditions for stem cell expansion*' section. As a step forward, hPSC cultures subsequently utilized feeder layer conditioned medium (CM). Next, serum-free medium

Figure 1

(a)

Platforms	Initial properties	Recent advances	Remaining challenges
2D 	• Easy visualization of morphology • Low expansion rate • Poor scalability	• Defined, xeno-free, GMP compatible [31] • High expansion rate [21] • Demonstrated small-scale long-term culture [25,44]	• Automation and scale-up are difficult • Pluripotency loss, genetic instability, and karyotypic abnormality after long-term serial passaging • Low viability passaging and harvesting
Microcarriers 	• Scalable • High expansion rate • High surface area to volume ratio • Compatible with bioreactor technology • Potentially difficult cell harvest	• Defined, xeno-free, GMP compatible [65,69] • Identification and optimization of culture parameters important for hPSC expansion [56-58]	• Agglomeration related differentiation, and agitation induced shear and differentiation • Long seeding times • Low viability passaging and harvesting • Unverified potential for long-term, large-scale serial passaging • Unverified potential for large-scale automation
Self-aggregates 	• Scalable • High expansion rate • High surface area to volume ratio • Compatible with bioreactor technology	• Defined, xeno-free, GMP compatible [74] • Demonstrated long-term, large-scale serial passaging [71-74] • Eliminated need for agitation, and alleviated agglomeration and settling using polymer coculture [75,79,80,83]	• Diffusion limitations in aggregate core • Low viability passaging • Long preclustering times • Unverified potential for large-scale automation
Microencapsulation 	• Compatible with bioreactor technology • High surface area to volume ratio • Reduced agglomeration • Reduced agitation induced stress and differentiation • Potentially difficult cell harvest	• Defined, xeno-free, GMP compatible [91] • Demonstrated scalability [86,91,95] • High viability single-cell passaging and harvesting • High expansion rate [91]	• Diffusion limitations in aggregate core • Unverified potential for long-term, large-scale serial passaging • Unverified potential for large-scale automation

(b)



Platforms and media for hPSC expansion. **(a)** Common platforms for hPSC culture and expansion: initial characteristics, advances since 2010 and remaining challenges. **(b)** Evolution of culture media and substrates. Abbreviations: MEF, mouse embryonic fibroblasts; HFF, human foreskin fibroblasts; KSR, knockout serum replacement; BSA, bovine serum albumin; rh, recombinant human.

such as Knockout Serum Replacement (KSR) replaced CM and fetal bovine serum (FBS) [17], though KSR still contains animal-derived components. Subsequently, defined media such as mTesR1 (Stem Cell Technologies) or StemPro (ThermoFisher) enabled hPSC culture completely independent of feeder layers or CM; however, these formulations contain bovine serum albumin (BSA) and are thus not xeno-free. Finally, fully-defined, xeno-free media such as E8 (ThermoFisher and Stem Cell Technologies) and Nutristem-SF (Biological Industries) were developed to meet the media needs described in the '*Desirable culture conditions for stem cell expansion*' section and thereby facilitate controlled, reproducible large-scale expansion. Moreover, xeno-free formulations of KSR are also available (ThermoFisher). Extensive validation of these fully defined, xeno-free media with a range of hPSC lines is progressively emerging in the field.

Culture platforms

2D culture

2D culture was initially used for hPSCs [18] and to this day remains the most widely used format [19,20,21••,22,23••,24–26,27•,28,29•,30,31•,32–34]. Initially, 2D co-cultures (e.g., on MEFs) provided not only soluble but also ECM components [35], but as discussed above co-cultures are non-ideal. A major advance was the replacement of MEFs with Matrigel [36], though this laminin-rich mixture consists of hundreds of other animal-derived components [37], suffers from lot-to-lot variability, is difficult to scale-up, and can result in loss of pluripotency and abnormal karyotypes after repeated serial passaging [38,39]. While Matrigel-cultured hESCs have been utilized in human clinical trials [40], improved platforms are needed.

Recently, researchers have worked toward xeno-free and/or feeder-free culture substrates [22,41], which include human cell derived feeder layers [20,22,42], chemically defined peptide surfaces such as Synthemax [34], purified or recombinant proteins [31•,32,33,43], and fully-defined synthetic polymeric scaffolds [23••,28,29•,44,45]. Human feeder layers derived from human tumors [20], as well as human umbilical cord blood serum [26], have been shown to support long term hPSC culture without animal-derived substrates, though expansion rates on these platforms need to be explored. Synthemax was used as a fully-defined, xeno-free alternative to Matrigel, though initial reports indicated a modest ~4× fold expansion per 3–4 day passage, or a 41 h doubling time [34]. In contrast, hPSCs grown on defined, feeder-free substrates such as laminin 511 [43], nanofibrous gelatin [30], and nanocrystalline graphene [29•] demonstrated mean doubling times of ~30–37 h that are similar to Matrigel, still a standard in the field [27•,29•,30,43,44]. Furthermore, these new, defined substrates were able to maintain pluripotency over several month long culture, as assessed via a range of assays (Figure 2) [19,32,43,44]. Moreover, the ROCK inhibitor Y27632 (RI) has been shown to increase survival of dissociated hPSCs [46,47], thereby leading to high viability passaging [19,32]; however, viability of single-cell passage onto Matrigel is still low [32]. Miyazaki *et al.* showed that recombinant laminin in combination with RI interestingly support high viability, single-cell passage on 2D, achieving a 200-fold expansion in 30 days [32]. Additional studies could further confirm this trend.

2D substrates based on recombinant proteins or synthetic peptides represent significant biological and biotechnological advances, though economic considerations can challenge their scale-up [25]. As alternatives, fully-defined, synthetic substrates — including synthetic heparin polymers [44], nanocrystalline graphene [29•], polysulfone [21••] and synthetic zwitterionic polymer hydrogels [25] — may be more economical to produce on a larger scale, though preparation of some materials (e.g. nanocrystalline graphene) may be technically challenging. Synthetic substrates may also have lower lot-to-lot variability than Matrigel, and importantly for hPSC expansion, maintain hPSC pluripotency in long-term cultures [21••,29•]. In general, high serial expansion rates can be a major challenge for 2D cultures, with reports of 4–10× expansion per 4–6 day passage [19,27•,34] or mean doubling times of >35 h [29•,34,43,44] (Figure 2). Through serial passaging hPSCs as small clusters on a defined, 3,4-dihydroxy-L-phenylalanine (DOPA) coated polysulfone membrane with mTeSR1 medium [21••], however, Kandasamy *et al.* attained a 20–30 fold expansion per 6 day passage, a substantial improvement over conventional 2D culture. In other interesting progress toward synthetic substrates, Celiz *et al.* demonstrated that powerful high throughput screening techniques could be applied to ~1000 member combinatorial libraries of synthetic polymer candidates to identify an optimal substrate for efficient hPSC expansion using StemPro and mTeSR1 media [23••]. Moving forward, using fully defined, xeno-free media such as E8 and Nutristem with these recent advances will be beneficial.

Recent advances in 2D platforms have thus attained many desirable culture criteria outlined in the '*Desirable culture conditions for stem cell expansion*' section — fully defined media and substrate, GMP compatible systems capable of maintaining long-term pluripotency and genomic stability, and in some cases achieving high expansion rates — compared to poorly defined substrates [16]. However, 2D platforms may be difficult to scale up [48], and for example generating ~10^{13} cells needed to treat PD would require ~1,000,000 T75 flasks or ~10 soccer fields worth of cell culture area. Automation of 2D cultures is possible with technologies such as Cell Factory and accompanying robotic Cell Factory manipulators (ThermoFisher) [49,50]; however, such systems would still require substantial high quality laboratory space to generate clinical scales of hPSCs for many applications [51,52]. Also, to date most defined 2D culture platforms

Figure 2

(a)

2D substrates

Culture method	Cell lines	Base medium condition	Max. no of passages	Longest time in culture	Max culture volume (ml)	Fold expansion /time	Fold expansion /passage	Mean division time (h)	Pluripotency tests	Ref.
Vitronectin, cluster passage	hESCs: MEL1, MEL2, hES3	StemPro, without ascorbate	10	-	-	-	-	-	Karyotyping, EB, teratoma,	33
Laminin 511, cluster passage	hESCs: H1, H9, HS207, HS420, HS401	O3, H3	28	150-180d	-			~36	ICC, qPCR, WB, karyotyping, EB, teratoma	43
UV treated polystyrene, single cell passage	hESCs: BG01, WIBR1, WIBR3, hiPSCs	DMEM	>10	>150d	-	1250x/18d	10	-	ICC, karyotyping, teratoma	19
Laminin E8, single cell passage	hESC: H9	mTesR1	35	~175d	-	200x/30d	-	-	Flow, karyotyping, EB, teratoma	32
Porous PE membrance scaffolds coated with matrigel, cluster passage	hESC: H9	mTesR1	5	~15-20d	-			26	ICC, qPCR	28
Synthemax, cluster passage	hiPSCs: IMR90, GIBCO Episomal	mTesR1	12	120-150d	-	4x/3-4d	~4	41.2		34
Human tumor derived matrix, cluster passage	hESCs: FES 29, H9, hiPSCs: HEL 11.4	StemPro	15	-	-	-	-	-	ICC, qPCR, karyotyping, EB, directed neuronal and hepatocyte differentiation, teratoma	20
Hydrogel based matrix with synthetic heparin polymers, cluster passage	hESCs: HUES9, HUES6; hiPSC	StemPro	20	>240d	-	-	-	38	ICC, qPCR, karyotyping, EB	44
Vitronectin peptide decorated poly vinyl alcohol/hyaluran nanofibers, cluster passage	hiPSCs (Guanzhou Institutes of Biomedicine and Health)	mTesR1	0	5d	-	-	-	-	qPCR	24
Zwitterionic hydrogel, cluster passage	hiPSCs: human foreskin fibroblasts and human gingival fibroblasts derived	human cell conditioned medium (GlobalStem)	-	270d	-	170x/270d	-	-	ICC, qPCR, WB, karyotyping, EB, teratoma	25
Nanofibrous gelatin, cluster passage	hESCs: H1, H9; hiPSCs: (253G1)	mTesR1	20	~90d	-	-	-	~30	ICC, qPCR, flow cytometry, AP, karyotyping, EB, RNAseq	30
LN521, cluster passage	hESCs: HUES7; hiPSCs: human fibroblast derived	E8, Nutristem, mTeSR1	10	~50d	-	-	-	-	ICC, qPCR, AP, karyotyping, EB, teratoma	31
Polysulfone coated with DOPA, cluster passage	hESCs: Hues7, H1, H7; hiPSCs: hFib2-iPS4, iPS-IMR90-4	mTesR1	10	60d	-	20-30x/6d	20-30	-	ICC, qPCR, karyotyping, teratoma	21
Matrix derived from human umbilical cord blood serum, cluster passage	hESCs HN4; hiPSCs: DYP0530	KO-DMEM	10	~50d	-	-	-	-	ICC, qPCR, karyotyping, EB, teratoma	26
Synthetic polymeric material, cluster passage	hESC: HUES7; hiPSCs: BT1	StemPro, mTeSR1	5	15d	-	-	-	-	ICC, karyotyping, directed differentiation of germ layers	23
Vitronectin modified polymeric hydrogels, cluster or single cell passage	hESCs: WA09; hiPSCs: HPS0077	mTesR1, E8	20	-	-	-	~10-15	-	ICC, AP, EB, teratoma	27
Polymeric nanofibrous substrates, cluster passage	hESCs; hIPSCs: HES9-EOS, IPSC-C11	mTesR1	0	56d	-	-	-	-	ICC, qPCR, flow cytometry, karyotyping, EB	45
Graphene, cluster passage	hESCs:H9; hiPSCs:NSC derived	KO-DMEM	10	-	-	-	-	37	ICC, qPCR, karyotyping, EB, teratoma, RNAseq	29

(b)

3D microcarriers

Trimethyl ammonium coated polystyrene beads, static culture, single cell or cluster passage	hESCs: ESI-017	KO-DMEM	6	42d LT; 5d LS	80	14x/42d	3	-	ICC, qPCR, spontaneous and directed differentiation into 3 germ layers	54
MEF or Matrigel coated MC, dynamic suspension culture, single cell passage	hESCs: H1, H9	MEF CM	~10-11	>60d LT LS	60	-	-	35	ICC, flow cytometry, karyotyping, spontaneous differentiation into 3 germ layers	62
Matrigel coated MC, dynamic suspension culture, cluster passage	hESCs: HES2, HES3	CM, mTesR1, StemPro	25	180d LT; 49d LS	50	-	-	21	ICC, flowcytometry, karyotyping, EB, teratoma	56
Laminin and Vitronectin coated MC, static culture, cluster passage	hESCs: HES3, H7	StemPro	20	140d LT	6 well plate (2ml)	8.5x/7d	8.5	-	ICC, karyotyping, EB, teratoma	68
Vitronectin coated MC, dynamic suspension culture, single cell passage	hESCs: H9; hiPSCs: IMR90	mTeSR1, TeSR2	5	30d LT LS	50	24x/6d	24	-	ICC, flow cytometry, karyotyping, EB	63
Matrigel coated MC, dynamic suspension culture, cluster passage LT static	hiPSCs: IMR90	mTeSR1	10	70d LT; 7d LS	100	20x/7d	20	35.8	ICC, EB, karyotyping, directed differentiation	58
Trimethyl ammonium coated MC (Hillex II), dynamic suspension culture, cluster passage	hESCs: H9	KSR-XF, BRASTEM	0	11d LS	60	-	-	25.3	ICC, flow cytometry, EB	65
pLL, Vitronectin, Mouse Laminin coated MC,	hESCs: HES3, H7; hiPSCs: IMR90	mTeSR1	3	21d LS	50	15x/7d	15	-	ICC, flow cytometry, EB	64
Laminin 521 coated MC, dynamic suspension culture. single cell passage	hESCs: HES3	mTeSR1	10	70d LT; 7d LS	50	7.5x/7d	7.5	26	ICC, karyotyping, EB	57
Vitronectin coated MC, dynamic suspension culture, cluster passage	hiPSCs: Gibco CD34 derived	E8	0	7-11d LS	50	-	-	-	ICC, qPCR, flow cytometry, EB, directed differentiation, karyotyping	69

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Figure 2.

(c)

3D aggregates	Dynamic suspension culture, cluster passage	hESCs: I4, I5, I6, H9.2, H7, H9,H14; hiPSCs: iF4, J1.2.3, C3, C2, KTN7, KTR13	DMEM/F12 + KSR	58	>365d LT; 90d LS	25	25x/10d	-	35	ICC, flow cytometry, karyotyping, EB, teratomas	70
	Dynamic suspension culture, cluster passage	hESCs: HES1, HES2, H7	Neurobasal	10	70d LT	12 well(~2ml)	100x/35d	-	24	ICC, karyotypiing, EB, teratomas	84
	Dynamic suspension culture, single cell passage	hESCs: HES3; hIPSCs	mTesR1	20	80d LT LS	25	4-6x/4d	~4-6	-	ICC, qPCR, flow cytometry, EB, karyotyping, microarray	71
	Dynamic suspension culture, single cell passage	hESCs: H9	mTesR	3	21d LS	100	25x/6d	25	35	ICC, flow cytometry, karyotyping, teratoma	80
	Dynamic suspension culture, single cell passage	hESCs: hES2, hES3	mTesR1	5	35d LT; 7d LS	50	0.3-2x/7d	2	-	ICC, flow cytometry, karyotyping, directed cardiomyocyte differentiation, teratoma	81
	Dynamic suspension culture, single cell passage	hESCs: Royan H5 and Royan H6; hiPSCs: hiPSC1 and hiPSC4	DMEM/F12 +/- MEF CM	10	70-100 d LT LS	100	8x/7-10d	8	-	AP, flow, spontaneous differentiation	72
	Dynamic suspension culture, single cell passage	hESCs: H9	StemPro	21	64d LT LS	60	1.3e13x/60 d	4.3	29.3	Flow cytometry, karyotyping, EB, directed cardiomyocyte differentiation, teratoma	73
	Dynamic suspension culture, single cell passage with aggregate seeding	hiPSCs: hCBiPSC2	mTesR1	1	7d LS	100	5.5x/7d	5.5	-	ICC, flow cytometry, spontaneous differentiation	76
	Dynamic suspension culture, single cell passage	hiPSCs: TNC1, BC1	E8	25	~75d LT, LS	100ml capacity	2.4-3.5x/3-4d	2.4-3.5	-	ICC, flow cytometry, karyotyping, EB, teratoma	74
	Dynamic suspension culture with thermoresponsive worms, cluster passage	hESCs: H9; MEL1, MEL2, NKX2-5	AEL, Stempro hESC SFM	-	18d LT	-	30x/18d	3	-	Flow cytometry, qPCR, karyotyping, EB	83
	Dynamic suspension, cluster passage	hESCs: Khes-1, H9, 253 G1, HES3	mTesR	8	40d LT; 5d LS	2.00E+02	1e6-1e7x/40d	~10-20	-	ICC, karyotyping, EB, teratoma	79

(d)

3D microencapsulation	Encapsulation in alginate capsules, continuous culture	hESCs: H1	DMEM ,KSR	0	260d LT, no passage	-	-	-	-	ICC, qPCR, differentiation to neurons and chrondocytes	85
	Encapsulation of single cells in alginate beads	hESCs: H9	KSR, MEF-CM	1	15d LS	125ml capacity	9x/15d	9	-	Directed cardiomyocyte differentiation	95
	Encapsulation of microcarriers in alginiate beads	hESCs: SCED461	MEF-CM	1	20d LS	100	20x/20d	20	-	ICC, flow, EB	86
	Alginate microfibers, small cluster passaging after enzyme digestion of gel	hESCs: BGO1V, HUES7; hiPSCs: hFib2-iPS4, PD-iPS5	mTeSR1	10	60d LT	-	10x/ 6d	10	27-34	AP, flow cytometry, karyotyping, spontaneous differentiation, teratoma	93
	Encapsulation of single cells in pNIPAAm-PEG hydrogels, single cell or cluster passage	hESCs: H1, H9; hiPSCs: iPS-MSC, iPS-Fib2	E8	60	280d LT; 5d LS	60	10-20x/4 or 5d	20	-	ICC, karyotyping, EB, directed differentiation to dopaminergic neuron progenitors, cardiomyocytes, endoderm progenitors, teratoma	91

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(Figure 2. Continued) Summary of recent hPSC expansion technology. **(a)** 2D substrates **(b)** 3D microcarriers **(c)** 3D aggregates and **(d)** 3D microencapsulation. Abbreviations: CM, conditioned medium; LT, long term serial passage; d, days; m, months; LS, large-scale expansion model in stirred flasks; ICC, Immunocytochemistry; AP, alkaline phosphatase activity; EB, embryoid body. Values for the different columns, when not explicitly stated within the cited article, were calculated when possible based on the available data. Longest times in culture were distinguished between long-term culture (LT), and large-scale expansion (LS).

involve relatively modest expansion rates (e.g., ~4–10× per passage, Figure 2), can entail potentially harsh mechanical or enzymatic passaging that can compromise cell viability [19], and are associated with inhomogeneous rates of cluster growth. Further work is needed to address these challenges, particularly as hPSC-based therapies progress toward larger clinical trials and eventually to commercialization.

3D culture platforms

3D microcarrier-based

A long-standing approach in bioprocess engineering — such as for recombinant protein and monoclonal antibody production — is to scale up to 3D cultures rather than scale out on 2D surfaces [53]. hPSC cultures were first transitioned from 2D surfaces to 3D by using microcarriers (MCs) [54], which offered a significant increase in surface area to culture volume ratio over 2D platforms [48,55] and thus offered the potential for considerable savings in consumable resources and cost-effective scale up [56,57•]. For example, a 100 ml culture with 0.3 g of cytodex 1 (~1e6 MCs) can provide as much surface area as 20 T75 flasks. With this 2–6-fold increase in yield (i.e., the number of cells generated per volume of culture medium) compared to 2D cultures [56,57•,58], MC-based suspension cultures have been reported to significantly reduce matrix and medium costs [57•]. Additionally, MCs are compatible with dynamic (stirred or perfusion reactors) cultures [59•], which are often used for biomanufacturing and have thus been well-developed for efficient, automated

scale-up with real-time monitoring of parameters (e.g., oxygen concentration, pH) important for hPSC expansion [55,60]. Continuous removal of metabolic byproducts, inhibitory cytokines, or pro-differentiation autocrine factors in continually perfused dynamic cultures may also aid the expansion of pluripotent stem cells [61] and mitigate DNA damage, genetic instability, and karyotypic abnormality [38]. Thus, MC cultures scaled up to 50–100 ml batch spinner flasks allowed a 20–24×-fold expansion in some instances and maintained ~80% pluripotency and genetic stability for several weeks [56,58,62,63].

However, MC cultures have faced several challenges. For example, MCs were initially coated with poorly defined feeder layers (e.g. MEFs) [62] or animal-derived substrates (e.g., Matrigel, or animal derived laminin) [56,58,62,64]. Also, low initial cell attachment and long incubation times for hPSC seeding on MCs (termed the seeding or adaptation period) [62,65], agglomeration and agitation related stress [62,66], and agitation-induced differentiation [58,67] generally reduce the effectiveness of these platforms for hPSC expansion. Agitation rate is an especially important parameter that must be appropriately tuned if possible to balance between agglomeration and aggregate settling, and shear stress related cell damage.

Recent developments have addressed several of these concerns and thereby improved MC-based hPSC suspension culture. In parallel with advances discussed in the '*2D culture*' section above for 2D platforms, fully defined, xeno-free substrates such as recombinant vitronectin and laminin have been used for coating MCs [48,57•,65,68,69•]. Additionally, the use of defined, adhesion-promoting laminin or vitronectin derived peptide coatings significantly increased hPSC attachment and reduced seeding time [57•,63]. Interestingly, defined coatings also resulted in faster hPSC doubling times [57•,65] compared to Matrigel or MEF coatings [58,62]. For example, using vitronectin-coated MCs and single-cell passaging, Fan *et al.* reported a 24-fold expansion per passage and successful culture in dynamic suspension for a month [63]. Furthermore, MC culture parameters that may further increase hPSC expansion efficiency — such as agitation speed, initial static culture period, cell seeding density, and feeding rate — were identified and optimized [56,57•,58]. For instance, Bardy *et al.* effectively doubled the hPSC expansion rate and obtained a 20× fold expansion over 7 days using MC cultures in stirred spinner flasks by doubling the feeding rate [58].

However, MCs encounter at least some of the disadvantages of 2D cultures. For example, cells must be chemically or mechanically harvested from MCs [69•], which reduces cell viability, lowers yield, and is a particularly difficult process for porous beads [48]. Furthermore, extended, long-term, dynamic suspension culture has not been demonstrated with MCs, as most studies report expansion for a few weeks [54,57•,58,65,69•]. Furthermore, demonstrating compatibility with automation technology will be particularly useful. Addressing these remaining challenges will encourage wider use of this promising, well-documented technology.

Self aggregates

Another well-established 3D format is suspension culture, where hPSCs are suspended as dissociated single cells, preformed aggregates, or small clusters and propagated under static or dynamic conditions. Similarly to MC cultures described in the '*3D microcarrier-based*' section, aggregate cultures can also utilize bioreactor technology, facilitating efficient volumetric scale-up. Two main benefits of suspension culture over MCs are: the demonstrated capacity for extended long-term passaging in dynamic cultures, which are ultimately necessary for bioreactor mediated scale-up [70], and the lack of need for a static seeding/adaptation period [71]. hPSCs cultured in dynamic suspension in stirred flasks have reportedly maintained pluripotency and genetic stability for months [70–74], while attaining a modest 2–8-fold expansion per passage every 4–7 days [71–74].

3D aggregate cultures initially faced several challenges. This included reduction in cell viability due to shear-related stress from agitation or from surface foaming [75], as well as agglomeration and settling of cell clusters [76,77]. Also, large clusters formed that posed diffusion limitations, uncontrolled differentiation, and necrosis in the aggregate cores [70,78]. Furthermore, expansion rates were reduced possibly due to the acidic conditions and reduced oxygen concentrations that accompany rapid metabolism [76]. Finally, there was a need for initial aggregate formation, requiring long pre-clustering times, before inoculating large-scale dynamic cultures.

Several approaches were suggested to solve these issues, including addition of anti-foaming agents such as pluronic to reduce bubbling related shear stress at the air-medium interface [75], both addition of viscous polymers to reduce aggregate settling and culturing within gas-permeable membranes to minimize the need for stirring [79••], steric hindrance agents to reduce aggregation [79••], and repeated frequent passaging as single cells to control aggregate growth [80]. These advances increased proliferation rates in 3D suspension cultures, achieving 20–25-fold expansion per 5–6 days passage for specific cell lines [79••,80]. Furthermore, addition of ROCK inhibitor to culture medium, as discussed earlier in the '*Culture medium*' section, facilitated passaging as single cells and thereby allowed more homogenous, controlled growth rates [70,75,81,82]. In a unique approach, Chen *et al.* mixed dissociated hPSCs and a vitronectin decorated thermoresponsive polymer to rapidly generate hPSC-polymer aggregates. Suspension

culture of these aggregates avoided the need for long pre-clustering times and resulted in enhanced expansion in suspension culture compared to hPSC self-aggregates [83••].

Recent technologies have thus addressed many, but not all, of the initial challenges listed above for suspension culture. Persistent problems include undesirable necrosis and uncontrolled differentiation within larger aggregates and the need for aggregation before initiating large-scale cultures. Furthermore, dissociated hPSCs do not survive well in suspension culture (even in the presence of ROCK inhibitor), with <50% post-passage cell viabilities in some cases [81,82,84]. Demonstrating the potential for automation is also needed. Future advances may further increase the applicability of this culture format for large-scale hPSC expansion.

3D microencapsulation

Another 3D culture method is hPSCs microencapsulation into polymer matrices such as alginate [85,86], hyaluronic acid (HA) [87], chitosan-alginate complex [88] or poly (lactic-co-glycolic acid)-poly(L-lactic acid) copolymers [89,90]. In this method, hPSCs are homogenously mixed with a polymer(s) as single cells or as small aggregates, and a polymer gelation process then encapsulates the cells into polymeric particles, for example ones shaped as spheres or 'worms' [86,91–93]. In addition to benefiting from the general advantages of 3D culture, microencapsulation reduces both agglomeration and the adverse effects of agitation related shear [86,91]. Moreover, microencapsulation within a solid phase material offers the potential for precise control over multiple features of the cellular microenvironment, such as tuning biochemical and mechanical cues to mimic those of the natural extracellular environment [94].

Several challenges faced by microencapsulation are: efficient retrieval of cells from the polymer matrix at the end of a culture, which can entail harsh enzymatic or mechanical treatments detrimental to cell viability [53], the need for validation with fully defined media, and potential diffusion limitation within a polymer gel that could affect differentiation and survival, which is an issue also faced by 3D aggregate cultures. To address the diffusion limitation problem, aggregates need to be regularly passaged as single cells or small aggregates [80], similar to 3D aggregate cultures. Thus, it is crucial for the polymer system to be amenable to easy, repeated passaging. PLGA is a convenient matrix for hPSC growth and differentiation for subsequent transplantation, but does not readily allow serial passaging for expansion [90]. In contrast, cells have been successfully retrieved from HA matrices with >70% viability; but the typical enzymatic treatment required for cell extraction may be detrimental to cell viability [87]. Similarly, cells encapsulated within alginate capsules were retrieved by the simple addition of a chelating agent, making this platform effective for cell harvest [85,86], though of cell viability post-harvest was not reported. Additionally, repeated, serial passaging of hPSCs encapsulated within HA or alginate beads has not been demonstrated, passaging as single cells in 3D encapsulated polymer matrixes have typically resulted in significant reductions in cell viability, and fully-defined media have not yet been tested with such microencapsulation materials [85,86,95].

The recent application of stimuli-responsive polymers to 3D culture of stem cells has addressed several remaining challenges [91]. As discussed earlier, one challenge in 3D aggregate culture is diffusion limitation, requiring frequent passaging of hPSC clusters as single cells. We recently found that thermoresponsive polymers such as poly-N-isopropyl-poly-acrylamide (pNIPAAm) facilitate cell retrieval without harsh mechanical or chemical treatment to dissociate the gel and thereby allows repeated, high viability cell passaging and final cell harvest [91]. Another potential drawback of 3D aggregate suspension cultures is the need for reaggregation following single cell passage, which not only increases process time but leads to heterogeneous cluster formation and issues associated with large aggregates. In contrast, pNIPAAm-PEG efficiently supported single cell passaging [91], thereby eliminating the need for reaggregation and benefiting from clonal expansion. Repeated, high viability, single-cell passaging combined with high expansion rates of ~20-fold per passage led to $\sim 10^{72}$ fold expansion over 60 passages within the thermoresponsive platform in fully-defined medium, and the cells maintained high levels of pluripotency and genetic stability [91]. Subsequent work indicated that the thermoresponsive polymer compared favorably to hPSC culture in suspension as self-aggregates or encapsulated within alginate or agarose beads [91,96••]. Furthermore, we have found that hPSCs encapsulated within this polymer can be differentiated into a range of functional cell types for a variety of applications, including midbrain dopaminergic neurons and oligodendrocyte progenitor cells for regenerative medicine in PD and spinal cord injury respectively (Adil, Rodrigues *et al.*, submitted). Moving forward, demonstration of automated long-term maintenance of material-encapsulated hPSC cultures should facilitate scale-up of this promising technology.

Conclusion

We have discussed recent advances in culture medium and platform technologies for hPSC expansion. While hPSC culture conditions initially included undefined components of animal origin, involved modest expansion rates, and maintained pluripotency to a limited extent after long-term expansion, recent advances in both the soluble and solid phases of culture systems have enabled effective large-scale hPSC expansion can be achieved under

completely defined, xeno-free conditions (Figure 2). The current capabilities of the different platforms are summarized in Figure 1, and resolving the remaining challenges listed in the '*2D culture*' and '*3D culture platforms*' sections may further facilitate effective hPSC expansion.

Knowledge accumulated from different culture systems may inform future platform design and help realize hPSC expansion platforms that address a range of desirable criteria. For example the recent defined, xeno-free polymeric substrates developed for 2D cultures — in addition to methods to reduce agglomeration, cluster settling, and shear stress for 3D suspension cultures — may be adapted to improve 3D MC cultures. Moreover, coating MCs with cell-adhesive ligand modified (Section '*2D culture*') or stimuli-responsive polymers (Sections '*3D microcarrier-based*' and '*Self aggregates*') may also prove beneficial, especially during cell harvest as demonstrated for other adherent cell lines [97,98].

Moving forward, more stringent quality assessment of hPSC expansion products may prove useful. As discussed in the '*Desirable culture conditions for stem cell expansion*' section, in addition to high expansion rates, the end product of hPSC expansion should maintain a high level of pluripotency and genetic stability. Recently genetic variability in hPSCs and epigenetic changes following long-term culture have been reported [99,100]; however, many stem cell expansion studies do not include extensive tests of population variability (Figure 2). Current standards for quality control of stem cell expansion include immunocytochemistry and qPCR for a handful of pluripotency markers, embryoid body formation, and *in vivo* teratoma assays. In addition to these assays for pluripotency, more stringent monitoring of the stem cell fate during long-term culture and expansion will be needed as concepts progress toward products. Even recently, the most stringent pluripotency test applied is teratoma formation (Figure 2) [101]. Given that relatively small cell numbers are needed to form tumors in immunodeficient mice [101], this approach may not report the pluripotency of the entire hPSC population. While immunocytochemistry or flow cytometry can determine the fraction of the population expressing conventionally accepted pluripotency markers such as OCT4, SSEA1, and NANOG, it is challenging to use these techniques to effectively monitor the expression of (or identify) all the markers potentially responsible for population heterogeneity. Recently, total RNA expression of some hPSC expansion cultures has shed light on differentially expressed genes between different culture conditions [29•,30]. As a step toward improved quality control, incorporating assays to investigate a larger portion of the stem cell transcriptome rather than a few known pluripotency markers will be useful. Furthermore, higher resolution techniques such as single-cell RNA-seq [102,103] will undoubtedly offer future, deeper insights into population heterogeneity. Thus, eventual inclusion of single-cell transcriptome and epigenome analysis into the array of required pluripotency tests could conceivably better assess the quality of expanded hPSCs and further improve safety standards.

In summary, many of the challenges facing large-scale expansion of hPSCs have been addressed over the past several years, owing especially to advances made in 3D culture technologies. Moving forward, it will be key to efficiently automate these new technologies to facilitate reproducible, cost-effective scale-up to an industrial level, while in parallel reducing hPSC population heterogeneity by appropriately designing culture conditions informed by new stem cell biology knowledge.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Goldman SA: **Stem and progenitor cell-based therapy of the central nervous system: hopes, hype, and wishful thinking**. *Cell Stem Cell* 2016, **18**:174-188 http://dx.doi.org/10.1016/j.stem.2016.01.012.

2. Serra M, Brito C, Correia C, Alves PM: **Process engineering of human pluripotent stem cells for clinical application**. *Trends Biotechnol* 2012, **30**:350-359 http://dx.doi.org/10.1016/j.tibtech.2012.03.003.

3. Grskovic M, Javaherian A, Strulovici B, Daley GQ: **Induced pluripotent stem cells — opportunities for disease modelling and drug discovery**. *Nat Rev Drug Discov* 2011, **10**:915-929 http://dx.doi.org/10.1038/nrd3577.

4. Davila JC, Cezar GG, Thiede M, Strom S, Miki T, Trosko J: **Use and application of stem cells in toxicology**. *Toxicol Sci* 2004, **79**:214-223 http://dx.doi.org/10.1093/toxsci/kfh100.

5. Gjorevski N, Ranga A, Lutolf MP: **Bioengineering approaches to guide stem cell-based organogenesis**. *Development* 2014, **141**:1794-1804 http://dx.doi.org/10.1242/dev.101048.

6. Lindvall O: **Dopaminergic neurons for Parkinson's therapy**. *Nat Biotechnol* 2012, **30**:56-58 http://dx.doi.org/10.1038/nbt.2077.

7. Laflamme MA, Murry CE: **Regenerating the heart**. *Nat Biotechnol* 2005, **23**:845-856 http://dx.doi.org/10.1038/scientificamerican0203-24c.

8. Lock LT, Tzanakakis ES: **Stem/Progenitor cell sources of insulin-producing cells for the treatment of diabetes**. *Tissue Eng* 2007, **13**:1399-1412 http://dx.doi.org/10.1089/ten.2007.0047.

9. World Health Organization, 2016. (n.d.). http://www.who.int/en/.

10. Parkinson's Disease Foundation, 2016. (n.d.). http://www.pdf.org.

11. Badylak SF, Taylor D, Uygun K: **Whole organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds**. *Annu Rev Biomed Eng* 2011, **13**:27-53 http://dx.doi.org/10.1146/annurev-bioeng-071910-124743.

12. Desbordes SC, Studer L: **Adapting human pluripotent stem cells to high-throughput and high-content screening**. *Nat Protoc* 2013, **8**:111-130 http://dx.doi.org/10.1038/nprot.2012.139.

13. Watt FM, Huck WTS: **Role of the extracellular matrix in regulating stem cell fate**. *Nat Rev Mol Cell Biol* 2013, **14**:467-473 http://dx.doi.org/10.1038/nrm3620.

14. McDevitt TC, Palecek SP: **Innovation in the culture and derivation of pluripotent human stem cells**. *Curr Opin Biotechnol* 2008, **19**:527-533 http://dx.doi.org/10.1016/j.copbio.2008.08.005.

15. Chen KG, Mallon BS, McKay RDG, Robey PG: **Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics**. *Cell Stem Cell* 2014, **14**:13-26 http://dx.doi.org/10.1126/scisignal.2001449.Engineering.

16. Villa-Diaz LG, Ross AM, Lahann J, Krebsbach PH: **Concise review: the evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings**. *Stem Cells* 2013, **31**:1-7 http://dx.doi.org/10.1002/stem.1260.

17. Xu C, Rosler E, Jiang J, Lebkowski JS, Gold JD, O'Sullivan C, Delavan-Boorsma K, Mok M, Bronstein A, Carpenter MK: **Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium**. *Stem Cells* 2005, **23**:315-323 http://dx.doi.org/10.1634/stemcells.2004-0211.

18. Thomson J, Itskovitz-Eldor J, Shapiro S, Waknitz M, Swiergiel J, Marshall V, Jones J: **Embryonic stem cell lines derived from human blastocysts**. *Science* 1998, **282**:1145-1147 http://dx.doi.org/10.1126/science.282.5391.1145.

19. Saha K, Mei Y, Reisterer CM, Pyzocha NK, Yang J, Muffat J, Davies MC, Alexander MR, Langer R, Anderson DG, Jaenish R: **Surface-engineered substrates for improved human pluripotent stem cell culture under fully defined conditions**. *Proc Natl Acad Sci U S A* 2011, **108**:18714-18719 http://dx.doi.org/10.1073/pnas.1114854108.

20. Vuoristo S, Toivonen S, Weltner J, Mikkola M, Ustinov J, Trokovic R, Palgi J, Lund R, Tuuri T, Otonkoski T: **A novel feeder-free culture system for human pluripotent stem cell culture and induced pluripotent stem cell derivation**. *PLoS ONE* 2013, **8**:1-14 http://dx.doi.org/10.1371/journal.pone.0076205.

21. •• Kandasamy K, Narayanan K, Ni M, Du C, Wan ACA, Zink D: **Polysulfone membranes coated with polymerized 3, 4-dihydroxy-L-phenylalanine are a versatile and cost-effective synthetic substrate for defined long-term cultures of human pluripotent stem cells**. *Biomacromolecules* 2014, **15**:2067-2078.
In an exciting advance towards fully defined 2D substrates, hPSC grown on a 3,4-dihydroxy-L-phenylalanine (DOPA) coated polysulfone membrane resulted in a high 20–30-fold expansion every 6 day passage, a substantial improvement relative to prior 2D cultures.

22. Bergstrom R, Strom S, Holm F, Feki A, Hovatta O: **Xeno-free culture of human pluripotent stem cells**. *Human Pluripotent Stem Cells Methods Protoc*. 2011:399-409 http://dx.doi.org/10.1007/978-1-61779-201-4.

23. •• Celiz AD, Smith JGW, Patel AK, Hook AL, Rajamohan D, George VT, Flatt L, Patel MJ, Epa VC, Singh T, Langer R, Anderson DG, Allen ND, Hay DC, Winkler DA, Barrett DA, Davies MC, Young LE, Denning C, Alexander MR: **Discovery of a novel polymer for human pluripotent stem cell expansion and multilineage differentiation**. *Adv Mater* 2015, **27**:4006-4012 http://dx.doi.org/10.1002/adma.201501351.
In accordance with progressively increasing use of synthetic substrates for hPSC expansion, high throughput screening was applied to a library of ~1000 candidates to identify the first synthetic polymer capable of supporting hPSC culture and maintaining pluripotency.

24. Deng Y, Zhang X, Zhao Y, Liang S, Xu A, Gao X, Deng F, Fang J, Wei S: **Peptide-decorated polyvinyl alcohol/hyaluronan nanofibers for human induced pluripotent stem cell culture**. *Carbohydr Polym* 2014, **101**:36-39 http://dx.doi.org/10.1016/j.carbpol.2013.09.030.

25. Villa-Diaz LG, Kim JK, Lahann J, Krebsbach PH: **Derivation and long-term culture of transgene-free human induced pluripotent stem cells on synthetic substrates**. *Stem Cells Transl Med* 2014, **3**:1410-1417.

26. Ding Y, Yang H, Yu L, Xu CL, Zeng Y, Qiu Y, Li DS: **Feeder-free and xeno-free culture of human pluripotent stem cells using UCBS matrix**. *Cell Biol Int* 2015, **39**:1111-1119 http://dx.doi.org/10.1002/cbin.10484.

27. • Higuchi A, Kao S-H, Ling Q-D, Chen Y-M, Li H-F, Alarfaj AA, Munusamy MA, Murugan K, Chang S-C, Lee H-C, Hsu S-T, Kumar SS, Umezawa A: **Long-term xeno-free culture of human pluripotent stem cells on hydrogels with optimal elasticity**. *Sci Rep* 2015, **5** http://dx.doi.org/10.1038/srep18136.
The effect of substrate elasticity on hPSC growth was investigated by systematically varying stiffness of a vitronectin-modified hydrogel. Interestingly, stiff substrates with ~40 kPa storage modulus resulted in differenitation after 5 days of culture, while soft substrates with~20 kPa storage modulus maintained pluripotency.

28. Jin S, Yao H, Krisanarungson P, Haukas A, Ye K: **Porous membrane substrates offer better niches to enhance the wnt signaling and promote human embryonic stem cell growth and differentiation**. *Tissue Eng A* 2012, **18**:1419-1430 http://dx.doi.org/10.1089/ten.tea.2011.0474.

29. • Lee H, Nam D, Choi J-K, Arauzo-Bravo MJ, Kwon S-Y, Zaehres H, Lee T, Park CY, Kang H-W, Scholer HR, Kim JB: **Establishment of feeder-free culture system for human induced pluripotent stem cell on DAS nanocrystalline graphene**. *Sci Rep* 2016, **6** http://dx.doi.org/10.1038/srep18136.
The nanotopography, oxygen containing functional groups, and hydrophilicity of a diffusion-assisted synthesis-grown nanocrystalline graphene (DAS-NG) surface was sufficient to maintain hPSCs in long-term culture, without requiring an ECM-like biological coating.

30. Liu L, Yoshioka M, Nakajima M, Ogasawara A, Liu J, Hasegawa K, Li S, Zou J, Nakatsuji N, ichiro Kamei K, Chen Y: **Nanofibrous gelatin substrates for long-term expansion of human pluripotent stem cells**. *Biomaterials* 2014, **35**:6259-6267 http://dx.doi.org/10.1016/j.biomaterials.2014.04.024.

31. • Lu HF, Chai C, Lim TC, Leong MF, Lim JK, Gao S, Lim KL, Wan ACA: **A defined xeno-free and feeder-free culture system for the derivation, expansion and direct differentiation of transgene-free patient-specific induced pluripotent stem cells**. *Biomaterials* 2014, **35**:2816-2826 http://dx.doi.org/10.1016/j.biomaterials.2013.12.050.
In one of the first studies using fully defined, xeno-free culture systems on 2D, hPSCs expanded on a laminin 521 coated surface for 10 passages were demonstrated to maintained pluripotency and genetic stability.

32. Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, Hayashi M, Kumagai H, Nakatsuji N, Sekiguchi K, Kawase E: **Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells**. *Nat Commun* 2012, **3** http://dx.doi.org/10.1038/ncomms2231.

33. Prowse ABJ, Doran MR, Cooper-White JJ, Chong F, Munro TP, Fitzpatrick J, Chung TL, Haylock DN, Gray PP, Wolvetang EJ: **Long term culture of human embryonic stem cells on recombinant vitronectin in ascorbate free media**. *Biomaterials* 2010, **31**:8281-8288 http://dx.doi.org/10.1016/j.biomaterials.2010.07.037.

34. Jin S, Yao H, Weber JL, Melkoumian ZK, Ye K: **A synthetic, xeno-free peptide surface for expansion and directed differentiation of human induced pluripotent stem cells**. *PLoS ONE* 2012, **7** http://dx.doi.org/10.1371/journal.pone.0050880.

35. Soteriou D, Iskender B, Byron A, Humphries JD, Borg-Bartolo S, Haddock M-C, Baxter MA, Knight D, Humphries MJ, Kimber SJ: **Comparative proteomic analysis of supportive and unsupportive extracellular matrix substrates for human embryonic stem cell maintenance**. *J Biol Chem* 2013, **288**:18716-18731 http://dx.doi.org/10.1074/jbc.M113.463372.

36. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK: **Feeder-free growth of undifferentiated human embryonic stem cells**. *Nat Biotechnol* 2001, **19**:971-974 http://dx.doi.org/10.1038/nbt1001-971.

37. Hughes CS, Postovit LM, Lajoie GA: **Matrigel: a complex protein mixture required for optimal growth of cell culture**. *Proteomics* 2010, **10**:1886-1890 http://dx.doi.org/10.1002/pmic.200900758.

38. Jacobs K, Zambelli F, Mertzanidou A, Smolders I, Geens M, Nguyen HT, Barbé L, Sermon K, Spits C: **Higher-density culture in human embryonic stem cells results in DNA damage and genome instability**. *Stem Cell Rep* 2016, **6**:330-341 http://dx.doi.org/10.1016/j.stemcr.2016.01.015.

39. Villa-Diaz LG, Ross AM, Lahann J, Krebsbach PH: **The evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings**. *Stem Cells* 2014, **31**:1-7 http://dx.doi.org/10.1002/stem.1260.

40. Priest CA, Manley NC, Denham J, Wirth ED, Lebkowski JS: **Preclinical safety of human embryonic stem cell-derived oligodendrocyte progenitors supporting clinical trials in spinal cord injury**. *Regen Med* 2015, **10**:939-958 http://dx.doi.org/10.2217/rme.15.57.

41. Higuchi A, Ling Q-D, Ko Y-A, Chang Y, Umezawa A: **Biomaterials for the feeder-free culture of human embryonic stem cells and induced pluripotent stem cells**. *Chem Rev* 2011, **111**:3021-3035 http://dx.doi.org/10.1021/cr1003612.

42. Zou C, Chou B-K, Dowey SN, Tsang K, Huang X, Liu CF, Smith C, Yen J, Mali P, Zhang YA, Cheng L, Ye Z: **Efficient derivation and genetic modifications of human pluripotent stem cells on engineered human feeder cell lines**. *Stem Cells Dev* 2012, **21**:2298-2311 http://dx.doi.org/10.1089/scd.2011.0688.

43. Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, Hovatta O, Tryggvason K: **Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511**. *Nat Biotechnol* 2010, **28**:611-615 http://dx.doi.org/10.1038/nbt.1620.

44. Chang CW, Hwang Y, Brafman D, Hagan T, Phung C, Varghese S: **Engineering cell-material interfaces for long-term expansion of human pluripotent stem cells**. *Biomaterials* 2013, **34**:912-921 http://dx.doi.org/10.1016/j.biomaterials.2012.10.020.

45. Alamein MA, Wolvetang EJ, Ovchinnikov DA, Stephens S, Sanders K, Warnke PH: **Polymeric nanofibrous substrates stimulate pluripotent stem cells to form three-dimensional multilayered patty-like spheroids in feeder-free culture and maintain their pluripotency**. *J Tissue Eng Regen Med* 2015, **9**:1078-1083 http://dx.doi.org/10.1002/term.

46. Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K, Sasai Y: **A ROCK inhibitor permits survival of dissociated human embryonic stem cells**. *Nat Biotechnol* 2007, **25**:681-686 http://dx.doi.org/10.1038/nbt1310.

47. Xu Y, Zhu X, Hahm HS, Wei W, Hao E, Hayek A, Ding S: **Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules**. *Proc Natl Acad Sci U S A* 2010, **107**:8129-8134 http://dx.doi.org/10.1073/pnas.1002024107.

48. Chen AK-L, Reuveny S, Oh SKW: **Application of human mesenchymal and pluripotent stem cell microcarrier cultures in cellular therapy: achievements and future direction**. *Biotechnol Adv* 2013, **31**:1032-1046 http://dx.doi.org/10.1016/j.biotechadv.2013.03.006.

49. Terstegge S, Laufenberg I, Pochert J, Schenk S, Itskovitz-Eldor J, Endl E, Brustle O: **Automated maintenance of embryonic stem cell cultures**. *Biotechnol Bioeng* 2007, **96**:195-201 http://dx.doi.org/10.1002/bit21061.

50. Thomas RJ, Anderson D, Chandra A, Smith NM, Young LE, Williams D, Denning C: **Automated, scalable culture of human embryonic stem cells in feeder-free conditions**. *Biotechnol Bioeng* 2009, **102**:1636-1644 http://dx.doi.org/10.1002/bit.22187.

51. Jenkins MJ, Farid SS: **Human pluripotent stem cell-derived products: advances towards robust, scalable and cost–effective manufacturing strategies**. *Biotechnol J* 2015, **10**:83-95 http://dx.doi.org/10.1002/biot.201400348.

52. Brandenberger R, Burger S, Campbell A, Fong T, Lapinska E, a Rowley J: **Cell therapy bioprocessing**. *Bioprocess Int* 2011, **9**:30-37.

53. McDevitt TC: **Scalable culture of human pluripotent stem cells in 3D**. *Proc Natl Acad Sci U S A* 2013, **110**:20852-20853 http://dx.doi.org/10.1073/pnas.1320575111.

54. Phillips BW, Horne R, Lay TS, Rust WL, Teck TT, Crook JM: **Attachment and growth of human embryonic stem cells on microcarriers**. *J Biotechnol* 2008, **138**:24-32 http://dx.doi.org/10.1016/j.jbiotec.2008.07.1997.

55. Lam ATL, Chen AKL, Ting SQP, Reuveny S, Oh SKW: **Integrated processes for expansion and differentiation of human pluripotent stem cells in suspended microcarriers cultures**. *Biochem Biophys Res Commun* 2016, **473**:764-768 http://dx.doi.org/10.1016/j.bbrc.2015.09.079.

56. Oh SKW, Chen AK, Mok Y, Chen X, Lim UM, Chin A, Choo ABH, Reuveny S: **Long-term microcarrier suspension cultures of human embryonic stem cells**. *Stem Cell Res* 2009, **2**:219-230 http://dx.doi.org/10.1016/j.scr.2009.02.005.

57. • Lam AT-L, Li J, Chen AK-L, Birch WR, Reuveny S, Oh SK-W: **Improved human pluripotent stem cell attachment and spreading on xeno-free laminin-521-coated microcarriers results in efficient growth in agitated cultures**. *Biores Open Access* 2015, **4**:242-257 http://dx.doi.org/10.1089/biores.2015.0010.
Addressing previous challenges related to poor cell seeding and long cell attachment periods, laminin 521 coated microcarriers enabled efficient cell attachment and achieved rapid hPSC expansion.

58. Bardy J, Chen AK, Lim YM, Wu S, Wei S, Weiping H, Chan K, Reuveny S, Oh SKW: **Microcarrier suspension cultures for high-density expansion and differentiation of human pluripotent stem cells to neural progenitor cells**. *Tissue Eng C Methods* 2012, **19** http://dx.doi.org/10.1089/ten.tec.2012.0146 120904064742009.

59. • Want AJ, Nienow AW, Hewitt CJ, Coopman K: **Large-scale expansion and exploitation of pluripotent stem cells for regenerative medicine purposes: beyond the T flask**. *Regen Med* 2012, **7**:71-84 http://dx.doi.org/10.2217/rme.11.101.

60. Nienow AW: **Reactor engineering in large scale animal cell culture**. *Cytotechnology* 2006, **50**:9-33 http://dx.doi.org/10.1007/s10616-006-9005-8.

61. King JA, Miller WM: **Bioreactor development for stem cell expansion and controlled differentiation**. *Curr Opin Chem Biol* 2007, **11**:394-398 http://dx.doi.org/10.1016/j.cbpa.2007.05.034.

62. Nie Y, Bergendahl V, Hei DJ, Jones JM, Palecek SP: **Scalable culture and cryopreservation of human embryonic stem cells on microcarriers**. *Biotechnol Prog* 2009, **25**:20-31 http://dx.doi.org/10.1002/btpr.110.

63. Fan Y, Hsiung M, Cheng C, Tzanakakis ES: **Facile engineering of xeno-free microcarriers for the scalable cultivation of human pluripotent stem cells in stirred suspension**. *Tissue Eng A* 2013, **20**:1-43 http://dx.doi.org/10.1089/ten.TEA. 2013.0219.

64. Lam AT-L, Li J, Chen AK-L, Reuveny S, Oh SK-W, Birch WR: **Cationic surface charge combined with either vitronectin or laminin dictates the evolution of human embryonic stem cells/microcarrier aggregates and cell growth in agitated cultures**. *Stem Cells Dev* 2014, **23**:1688-1703 http://dx.doi.org/10.1089/scd.2013.0645.

65. Marinho PAN, Vareschini DT, Gomes IC, Paulsen BDS, Furtado DR, Castilho LDR, Rehen SK: **Xeno-free production of human embryonic stem cells in stirred microcarrier systems using a novel animal/human-component-free medium**. *Tissue Eng C Methods* 2012, **19** http://dx.doi.org/10.1089/ten.tec.2012.0141 121016064607007.

66. Chen AK-L, Chen X, Choo ABH, Reuveny S, Oh SKW: **Critical microcarrier properties affecting the expansion of undifferentiated human embryonic stem cells**. *Stem Cell Res* 2011, **7**:97-111 http://dx.doi.org/10.1016/j.scr.2011.04.007.

67. Leung HW, Chen A, Choo ABH, Reuveny S, Oh SKW: **Agitation can induce differentiation of human pluripotent stem cells in microcarrier cultures**. *Tissue Eng C Methods* 2011, **17**:165-172 http://dx.doi.org/10.1089/ten.tec.2010.0320.

68. Heng BC, Li J, Chen AK, Reuveny S, Cool SM, Birch WR, Oh SK: **Translating human embryonic stem cells from 2-dimensional to 3-dimensional cultures in a defined medium on laminin- and vitronectin-coated surfaces**. *Stem Cells Dev* 2012, **21**:1701-1715 http://dx.doi.org/10.1089/scd.2011.0509.

69. • Badenes SM, Fernandes TG, Cordeiro CSM, Boucher S, Kuninger D, Vemuri MC, Diogo MM, Cabral JMS: **Defined essential 8 medium and vitronectin efficiently support scalable xeno-free expansion of human induced pluripotent**

stem cells in stirred microcarrier culture systems. *PLOS ONE* 2016, **11**:1-19 http://dx.doi.org/10.1371/journal.pone.0151264.
This study constitutes one of the first demonstrations of hPSC expansion using fully defined, xeno-free conditions for 3D microcarriers.

70. Amit M, Chebath J, Margulets V, Laevsky I, Miropolsky Y, Shariki K, Peri M, Blais I, Slutsky G, Revel M, Itskovitz-Eldor J: **Suspension culture of undifferentiated human embryonic and induced pluripotent stem cells**. *Stem Cell Rev Rep* 2010, **6**:248-259 http://dx.doi.org/10.1007/s12015-010-9149-y.

71. Olmer R, Haase A, Merkert S, Cui W, Paleček J, Ran C, Kirschning A, Scheper T, Glage S, Miller K, Curnow EC, Hayes ES, Martin U: **Long term expansion of undifferentiated human iPS and ES cells in suspension culture using a defined medium**. *Stem Cell Res* 2010, **5**:51-64 http://dx.doi.org/10.1016/j.scr.2010.03.005.

72. Abbasalizadeh S, Larijani MR, Samadian A, Baharvand H: **Bioprocess development for mass production of size-controlled human pluripotent stem cell aggregates in stirred suspension bioreactor**. *Tissue Eng C Methods* 2012, **18**:831-851 http://dx.doi.org/10.1089/ten.tec.2012.0161.

73. Chen VC, Couture SM, Ye J, Lin Z, Hua G, Huang H-IP, Wu J, Hsu D, Carpenter MK, Couture LA: **Scalable GMP compliant suspension culture system for human ES cells**. *Stem Cell Res* 2012, **8**:388-402 http://dx.doi.org/10.1016/j.scr.2012.02.001.

74. Wang Y, Chou BK, Dowey S, He C, Gerecht S, Cheng L: **Scalable expansion of human induced pluripotent stem cells in the defined xeno-free E8 medium under adherent and suspension culture conditions**. *Stem Cell Res* 2013, **11**:1103-1116 http://dx.doi.org/10.1016/j.scr.2013.07.011.

75. Kehoe DE, Jing D, Lock LT, Tzanakakis ES: **Scalable stirred-suspension bioreactor culture of human pluripotent stem cells**. *Tissue Eng A* 2010, **16**:405-421 http://dx.doi.org/10.1089/ten.tea.2009.0454.

76. Olmer R, Lange A, Selzer S, Kasper C, Haverich A, Martin U, Zweigerdt R: **Suspension culture of human pluripotent stem cells in controlled, stirred bioreactors**. *Tissue Eng C Methods* 2012, **18**:772-784 http://dx.doi.org/10.1089/ten.tec.2011.0717.

77. Zweigerdt R, Olmer R, Singh H, Haverich A, Martin U: **Scalable expansion of human pluripotent stem cells in suspension culture**. *Nat Protoc* 2011, **6**:689-700 http://dx.doi.org/10.1038/nprot.2011.318.

78. Wu J, Rostami MR, Cadavid Olaya DP, Tzanakakis ES: **Oxygen transport and stem cell aggregation in stirred-suspension bioreactor cultures**. *PLOS ONE* 2014, **9**:1-12 http://dx.doi.org/10.1371/journal.pone.0102486.

79. •• Otsuji TG, Bin J, Yoshimura A, Tomura M, Tateyama D, Minami I, Yoshikawa Y, Aiba K, Heuser JE, Nishino T, Hasegawa K, Nakatsuji N: **A 3D sphere culture system containing functional polymers for large-scale human pluripotent stem cell production**. *Stem Cell Rep* 2014, **2**:734-745 http://dx.doi.org/10.1016/j.stemcr.2014.03.012.
Adding viscous polymers to 3D suspension cultures in gas permeable bags reduced aggregate settling and the need for continuous stirring, thereby reducing shear-related stress and facilitating high viability culture.

80. Krawetz R, Taiani JT, Liu S, Meng G, Li X, Kallos MS, Rancourt DE: **Large-scale expansion of pluripotent human embryonic stem cells in stirred-suspension bioreactors**. *Tissue Eng C Methods* 2010, **16**:573-582 http://dx.doi.org/10.1089/ten.tec.2009.0228.

81. Singh H, Mok P, Balakrishnan T, Rahmat SNB, Zweigerdt R: **Up-scaling single cell-inoculated suspension culture of human embryonic stem cells**. *Stem Cell Res* 2010, **4**:165-179 http://dx.doi.org/10.1016/j.scr.2010.03.001.

82. Olmer R, Haase A, Merkert S, Cui W, Paleček J, Ran C, Kirschning A, Scheper T, Glage S, Miller K, Curnow EC, Hayes ES, Martin U: **Long term expansion of undifferentiated human iPS and ES cells in suspension culture using a defined medium**. *Stem Cell Res* 2010, **5**:51-64 http://dx.doi.org/10.1016/j.scr.2010.03.005.

83. •• Chen X, Prowse ABJ, Jia Z, Tellier H, Munro TP, Gray PP, Monteiro MJ: **Thermoresponsive worms for expansion and release of human embryonic stem cells**. *Biomacromolecules* 2014, **15**:844-855.
Rapid aggregation mediated by mixing dissociated hPSCs with vitronectin-coated thermoresponsive polymers eliminated long pre-clustering times, which previously prevented convenient aggregate passaging. Additionally, simple cooling mediated liquefaction of the polymer system facilitated high viability cell retrieval and passage.

84. Steiner D, Khaner H, Cohen M, Even-Ram S, Gil Y, Itsykson P, Turetsky T, Idelson M, Aizenman E, Ram R, Berman-Zaken Y, Reubinoff B: **Derivation, propagation and controlled differentiation of human embryonic stem cells in suspension**. *Nat Biotechnol* 2010, **28**:361-364 http://dx.doi.org/10.1038/nbt.1616.

85. Siti-Ismail N, Bishop AE, Polak JM, Mantalaris A: **The benefit of human embryonic stem cell encapsulation for prolonged feeder-free maintenance**. *Biomaterials* 2008, **29**:3946-3952 http://dx.doi.org/10.1016/j.biomaterials.2008.04.027.

86. Serra M, Correia C, Malpique R, Brito C, Jensen J, Bjorquist P, Carrondo MJT, Alves PM: **Microencapsulation technology: a powerful tool for integrating expansion and cryopreservation of human embryonic stem cells**. *PLoS ONE* 2011, **6**:e23212 http://dx.doi.org/10.1371/journal.pone.0023212.

87. Gerecht S, Burdick JA, Ferreira LS, Townsend SA, Langer R: **Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells**. *Proc Natl Acad Sci U S A* 2007, **104**:1-6 http://dx.doi.org/10.1073/pnas.0703723104.

88. Li Z, Leung M, Hopper R, Ellenbogen R, Zhang M: **Feeder-free self-renewal of human embryonic stem cells in 3D porous natural polymer scaffolds**. *Biomaterials* 2010, **31**:404-412 http://dx.doi.org/10.1016/j.biomaterials.2009.09.070.

89. Liu Y, Fox V, Lei Y, Hu B, Il Joo K, Wang P: **Synthetic niches for differentiation of human embryonic stem cells bypassing embryoid body formation**. *J Biomed Mater Res – Part B Appl Biomater* 2014, **102**:1101-1112 http://dx.doi.org/10.1002/jbm.b.33092.

90. Levenberg S, Huang NF, Lavik E, Rogers AB, Itskovitz-Eldor J, Langer R: **Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds**. *Proc Natl Acad Sci U S A* 2003, **100**:12741-12746 http://dx.doi.org/10.1073/pnas.1735463100.

91. Lei Y, Schaffer DV: **A fully defined and scalable 3D culture system for human pluripotent stem cell expansion and differentiation**. *Proc Natl Acad Sci U S A* 2013, **110**:E5039-E5048 http://dx.doi.org/10.1073/pnas.1309408110.

92. Lou Y-R, Kanninen L, Kuisma T, Niklander J, Noon LA, Burks D, Urtti A, Yliperttula M: **The use of nanofibrillar cellulose hydrogel as a flexible three-dimensional model to culture human pluripotent stem cells**. *Stem Cells Dev* 2014, **23**:380-392 http://dx.doi.org/10.1089/scd.2013.0314.

93. Lu HF, Narayanan K, Lim SX, Gao S, Leong MF, Wan ACA: **A 3D microfibrous scaffold for long-term human pluripotent stem cell self-renewal under chemically defined conditions**. *Biomaterials* 2012, **33**:2419-2430 http://dx.doi.org/10.1016/j.biomaterials.2011.11.077.

94. Blow N: **Cell culture: building a better matrix**. *Nat Methods* 2009, **6**:619-622 http://dx.doi.org/10.1038/nmeth0809-619.

95. Jing D, Parikh A, Tzanakakis ES: **Cardiac cell generation from encapsulated embryonic stem cells in static and scalable culture systems**. *Cell Transpl* 2010, **19**:1397-1412 http://dx.doi.org/10.3727/096368910X513955.

96. •• Lei Y, Jeong D, Xiao J, Schaffer DV: **Developing defined and scalable 3D culture systems for culturing human pluripotent stem cells at high densities**. *Cell Mol Bioeng* 2014, **7**:172-183 http://dx.doi.org/10.1126/scisignal.2001449.Engineering.
hPSC encapsulation within a thermoresponsive hydrogel allowed high viability passaging as single cells and maintained high expansion rates for several months. This is a follow up to a prior study [91] that for the first time encapsulated hPSCs in a thermoresponsive polymer, and resolved several challenges facing microencapsulation cultures.

97. Tamura A, Kobayashi J, Yamato M, Okano T: **Temperature-responsive poly(N-isopropylacrylamide)-grafted**

microcarriers for large-scale non-invasive harvest of anchorage-dependent cells. *Biomaterials* 2012, **33**:3803-3812 http://dx.doi.org/10.1016/j.biomaterials.2012.01.060.

98. Yang HS, Jeon O, Bhang SH, Lee SH, Kim BS: **Suspension culture of mammalian cells using thermosensitive microcarrier that allows cell detachment without proteolytic enzyme treatment**. *Cell Transpl* 2010, **19**:1123-1132 http://dx.doi.org/10.3727/096368910X516664.

99. Wutz A: **Epigenetic alterations in human pluripotent stem cells: a tale of two cultures**. *Cell Stem Cell* 2012, **11**:9-15 http://dx.doi.org/10.1016/j.stem.2012.06.012.

100. Amps K, Andrews PW, Anyfantis G, Armstrong L, Avery S, Baharvand H, Baker J, Baker D, Munoz MB, Beil S, Benvenisty N, Ben-Yosef D, Biancotti J-C, Bosman A, Brena RM, Brison D, Caisander G, Camarasa MV, Chen J, Chiao E, Choi YM, Choo ABH, Collins D, Colman A, Crook JM, Daley GQ, Dalton A, De Sousa PA, Denning C, Downie J, Dvorak P, Montgomery KD, Feki A, Ford A, Fox V, Fraga AM, Frumkin T, Ge L, Gokhale PJ, Golan-Lev T, Gourabi H, Gropp M, Lu G, Hampl A, Harron K, Healy L, Herath W, Holm F, Hovatta O, Hyllner J, Inamdar MS, Irwanto AK, Ishii T, Jaconi M, Jin Y, Kimber S, Kiselev S, Knowles BB, Kopper O, Kukharenko V, Kuliev A, Lagarkova MA, Laird PW, Lako M, Laslett AL, Lavon N, Lee DR, Lee JE, Li C, Lim LS, Ludwig TE, Ma Y, Maltby E, Mateizel I, Mayshar Y, Mileikovsky M, Minger SL, Miyazaki T, Moon SY, Moore H, Mummery C, Nagy A, Nakatsuji N, Narwani K, Oh SKW, Oh SK, Olson C, Otonkoski T, Pan F, Park I-H, Pells S, Pera MF, Pereira LV, Qi O, Raj GS, Reubinoff B, Robins A, Robson P, Rossant J, Salekdeh GH, Schulz TC, Sermon K, Sheik Mohamed J, Shen H, Sherrer E, Sidhu K, Sivarajah S, Skottman H, Spits C, Stacey GN, Strehl R, Strelchenko N, Suemori H, Sun B, Suuronen R, Takahashi K, Tuuri T, Venu P, Verlinsky Y, Ward-van Oostwaard D, Weisenberger DJ, Wu Y, Yamanaka S, Young L, Zhou Q: **Screening ethnically diverse human embryonic stem cells identifies a chromosome 20 minimal amplicon conferring growth advantage**. *Nat Biotechnol* 2011, **29**:1132-1144 http://dx.doi.org/10.1038/nbt.2051.

101. Zhang WY, de Almeida PE, Wu JC: **Teratoma formation: a tool for monitoring pluripotency in stem cell research**. *Stembook*. 2012:1-14 http://dx.doi.org/10.1002/9780470151808.sc04a08s32.

102. Wen L, Tang F: **Single-cell sequencing in stem cell biology**. *Genome Biol* 2016, **17**:71 http://dx.doi.org/10.1186/s13059-016-0941-0.

103. Saliba AE, Westermann AJ, Gorski SA, Vogel J: **Single-cell RNA-seq: advances and future challenges**. *Nucleic Acids Res* 2014, **42**:8845-8860 http://dx.doi.org/10.1093/nar/gku555.